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GROWTH AND PATHOLOGY OF MELANOMA S91 IN MICE OF STRAINS dba, A, AND C[1]

By GLENN H. ALGIRE, *assistant surgeon (R), National Cancer Institute, National Institute of Health, United States Public Health Service*

Melanoma S91 is a malignant melanoma that originated in 1937 in a JAX-dba female mouse at the base of the tail. The dba is a pigmented strain, dilute brown in color. This melanoma was first found by Dr. A. M. Cloudman, of the Roscoe B. Jackson Memorial Laboratory. Cloudman (*1*) reported the successful transplantation of the tumor into A mice fostered by dba females, and its failure to develop when transplanted into unfostered strain A mice. When received at the National Cancer Institute November 15, 1939, it was in the twelfth-generation transplant in mice of the strain of origin. The tumor had been carried routinely in the dba strain for 2 years before the studies reported herein were started.

Melanomas in mice were described by Harding and Passey (*2*) in 1930 and by Cloudman (*3*) in 1941. Melanoma S91 was also considered briefly by Spangler, Murray, and Little (*4*) in 1942. These authors (*4*) reported that this tumor gave 100-percent-successful transplants into the strain of origin and practically 100-percent-negative results in another inbred strain, A. It has been successfully transferred to 62 percent of the albino segregates of the F_2 generation from reciprocal A × dba crosses. There are no reports of successful transplants of this tumor into other albino strains. The experiments reported here were undertaken in connection with other work, in which it seemed desirable to transfer this melanoma to albino mice.

Since no complete pathologic description of this tumor has been made and since it is used in this laboratory for microscopic studies of tumor growth in vivo (*5*), as well as in biochemical (*6*, *7*) and endocrinological investigations (*8*), the pathologic characteristics of its growth in A and C, both albino strains, and in strain dba are recorded here. This paper is also concerned with the derivation of a predominantly amelanotic melanoma growing in strain C mice.

TRANSPLANTATION EXPERIMENTS

Eight strain C mice were given subcutaneous inoculations of small pieces of the melanoma from a dba host. Inoculations were made into the side of the chest wall through a 12-gage trocar and by hypodermic syringe into the ear with a 24-gage needle.

In three of the eight mice transplantation was successful, one mouse developing in 3 months a large pigmented mass measuring 8 x 15 mm. at the side of the chest. This take was used for the subsequent transfers,

[1] The inbred strains of mice, dba, A, and C, were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

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Two mice, during the same period, developed small pigmented nodules in the ear, measuring 2 x 3 mm. All these growths were classified histologically as malignant melanoma. The tumor line has been carried through 14 generations in strain C mice, with successful growth in each generation after the first, of from 80 to 100 percent. Implantation has been successful following either subcutaneous or intraperitoneal injections of tumor tissue.

A later repetition of this experiment resulted in takes in five of nine strain C mice into which the tumor was transplanted from a dba host.

Eight strain A mice were given tumor transplants in the same way as the strain C. After a period of about 8 months, pigmented masses were found in the chest wall in two of these mice. One was 5 x 7, and the other 4 x 6 mm. Both of these growths were diagnosed pathologically as malignant melanoma. This tumor line has been carried through five generations in strain A mice with successful growth in 50 to 70 percent of the transplants into this strain.

A repetition of this experiment resulted in takes in 2 of 11 strain A mice into which the tumor was transplanted from a dba host.

No difficulty was experienced in transferring the melanoma from strain C into the dba strain of origin. In one experiment, 7 of 10 mice gave positive takes, and in a second, 12 of 13 were positive. All tumors attained a size 10 x 10 mm. or larger within 2 months.

In the course of the foregoing work, portions of some of the subcutaneous transplants in both the dilute brown and albino strains were observed to be grayish white in color. As a result of this observation, transplants were made of nonpigmented parts of the tumor growing in strain C mice into other animals of the same strain, in the hope of obtaining a completely amelanotic melanoma.

Two strain C mice were given subcutaneous injections of small pieces of the amelanotic portions of the melanoma from a C host. Injections were made into the side of the chest wall through a 12-gage trocar.

Transplantation was successful in one animal which in 2 months developed a grossly nonpigmented mass measuring 7 x 10 mm. at the side of the chest. This take was used for the subsequent transfers. Histologic sections showed that all except a few cells were nonpigmented. The tumor line has been carried through 7 tissue generations in strain C mice, 6 to 10 mice being used in each generation. Successful growth resulted in about 40 to 50 percent.

GROSS PATHOLOGY

The subcutaneous melanotic transplants into the side of the chest wall were firm and in the albino mice were seen through the intact skin as reddish-purple or black masses. Figure 1 shows the gross appearance of the tumor in the dba strain of origin and in the two albino strains. The larger tumors ultimately showed ulceration of the overlying skin. The tumors were discrete, with fairly sharp outlines. Section revealed tissue which was predominantly black or reddish purple, an occasional tumor showing small elongated foci of nonpigmented grayish tissue several millimeters in length. There was a sharp demarcation between the pigmented tumor mass and the surrounding tissues; usually one or two large blood vessels extended from the axilla along the chest wall and into the tumor.

Those mice that had received inoculations of tumor tissue into the ear showed a small focus of black pigment immediately following inoculation. This usually became smaller and disappeared; but in the few mice in which takes were successful, the original black area became somewhat enlarged and nodular. In none of these animals did the takes approximate the size of the positive takes along the chest wall in the same period of time.

In those animals in which intraperitoneal inoculation of tumor tissue was followed by successful takes, the tumors

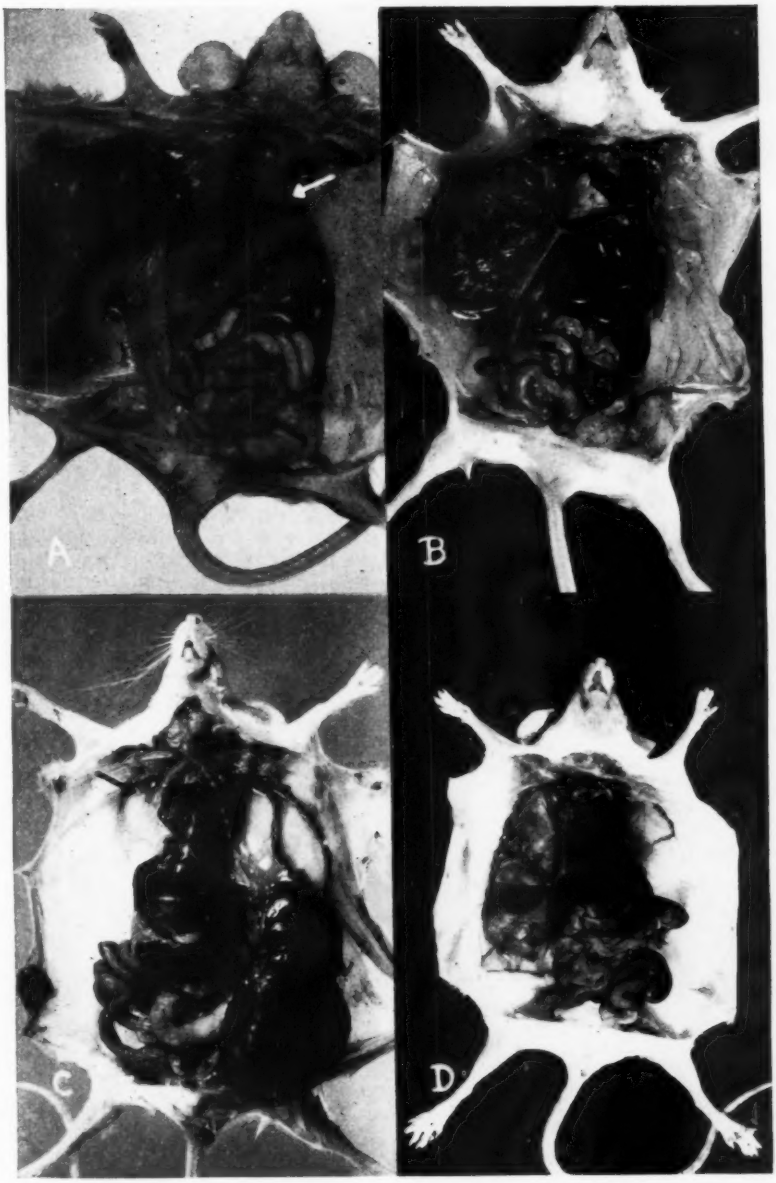

FIGURE 1.—Gross pathology of transplants. *A*, strain dba, in which tumor originated; *B*, strain A; *C*, strain C; *D*, amelanotic transplant in strain C. Note lung metastases in *A* and *C*.

were similar in appearance to the subcutaneous transfers, were often invasive, and reached rather large proportions.

The subcutaneous amelanotic transplants were firm and appeared to consist of nonpigmented grayish tissue. A section, however, revealed tissue which was grayish white at the periphery and black at the center (fig. 1, *D*). In successive tissue generations, the tumors became predominantly grayish white, with only a small central black area. So far, however, no completely amelanotic tumor has been found.

Metastases were observed grossly in practically all the albino mice that were carrying subcutaneous or intraperitoneal melanotic transplants of any apparent size. The metasases were most commonly observed in the lungs, but were also seen in the liver, kidney, adrenals, lymph nodes, and orbit in the region of the Harderian gland. They usually were multiple in any one organ and appeared as pure black or brown nodules measuring from pin-point size to 3 mm. each.

In only one instance, however, has a metastasis been found in the amelanotic strain. This was in the lung as a black nodule about 2 mm. in diameter.

MICROSCOPIC EXAMINATION[2]

The animals were killed by cervical dislocation. Sections of tumor for microscopic study were taken within a few minutes after death.

Routinely, the tissues were fixed in formaldehyde solution (1:10 U. S. P.). On occasion, Zenker's solution and Zenker-formol mixture were also used. Hematoxylin-and-eosin stain was employed routinely, and in addition the following stains were used in certain instances on suitably fixed specimens: Mallory's phosphotungstic acid-hematoxylin, the potassium ferrocyanide-hydrochloric acid test for iron, and Laidlaw's reticulum stain with nuclear counterstain.

The tumors of both pigmented and albino mice were indistinguishable histologically and were composed of elongated cells which showed a marked tendency to form twisting and interlacing bundles (fig. 2) as well as

[2] Dr. Jesse E. Edwards, of this Institute, examined the microscopic sections.

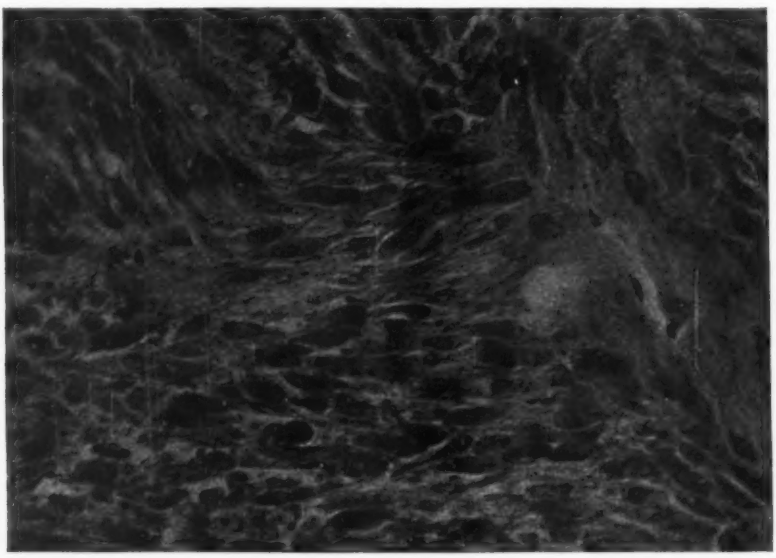

FIGURE 2.—Subcutaneous transplant showing elongated cells arranged in twisting and interlacing bundles. × 400.

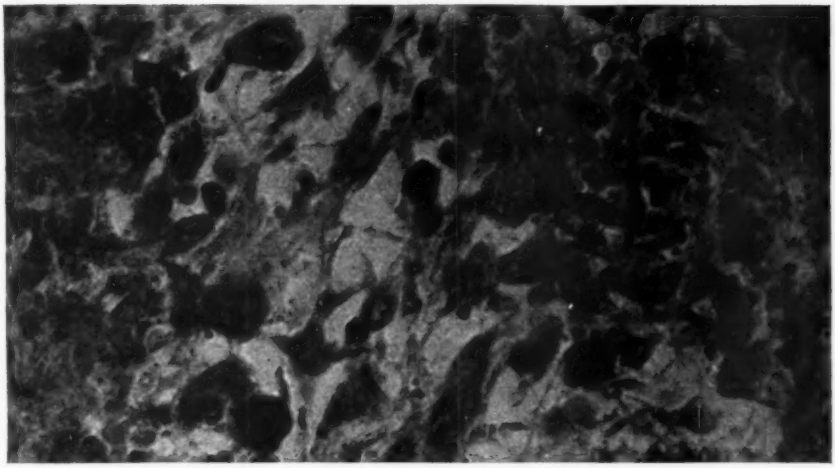

FIGURE 3.—Intracytoplasmic stippling of brown pigment granules. × 1000.

numerous small knots of tissue. The nuclei were relatively large, fusiform, or less commonly ovoid and vesicular, with the chromatin distributed unevenly. Most of the cells contained one to two small nucleoli. Mitotic figures were numerous. The cytoplasm had an irregular shape, but in most of the cells it was elongated, showing a faint eosinophilic stain. Many of the cells showed heavy intracytoplasmic stippling of brown pigment granules (fig. 3). The pigment cells were distributed irregularly, but some were found in practically every bundle or knot. The pigment failed to give a reaction for iron with the hydrochloric acid-potassium ferrocyanide test. Delicate strands of connective tissue penetrated among many of the cells. In sections stained with Mallory's phosphotungstic acid-hematoxylin stain, the cytoplasm contained numerous, delicate, wavy, purple fibrils which appeared to extend out of the cytoplasm into the intercellular spaces (fig. 4); with stains for demonstrating reticulum, the individual tumor cells were intimately associated with either delicate or heavy reticulum fibers (fig. 5).

The larger tumors showed varying amounts of necrotic tissue which usually lay in the center of the tumor. In the necrotic regions, it was common to observe collars of viable cells surrounding blood vessels. The actual necrotic tissue was as a rule highly pigmented, most of the pigment lying free, and a small amount within phagocytes.

Except for their almost complete lack of melanin pigment, the amelanotic tumors were indistinguishable from the melanoma described.

Microscopic examination confirmed the gross observations that metastases occurred in the lymph nodes, lungs, liver, adrenals, kidney, and orbital cavity. The neoplasm, growing as metastases, reproduced the structure of the transplants. Usually the pigmented cells were intermingled haphazardly; but in some instances, certain metastatic nodes were heavily pigmented, while others contained very little pigment (fig. 6). None of the latter were completely nonpigmented, however, for in these there were isolated tumor cells containing granules of pigment.

The metastatic tumors were usually destructive to the tissues in which they lay. However, in the case of certain of the smaller tumors the organ was

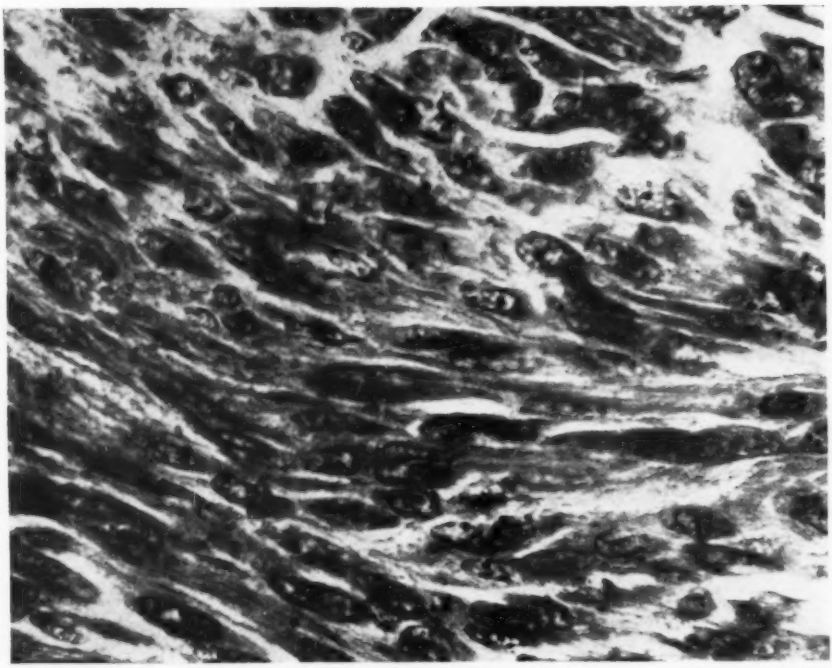

FIGURE 4.—Fibrils in close association with the cells which appear to extend out into the intercellular spaces. × 1350.

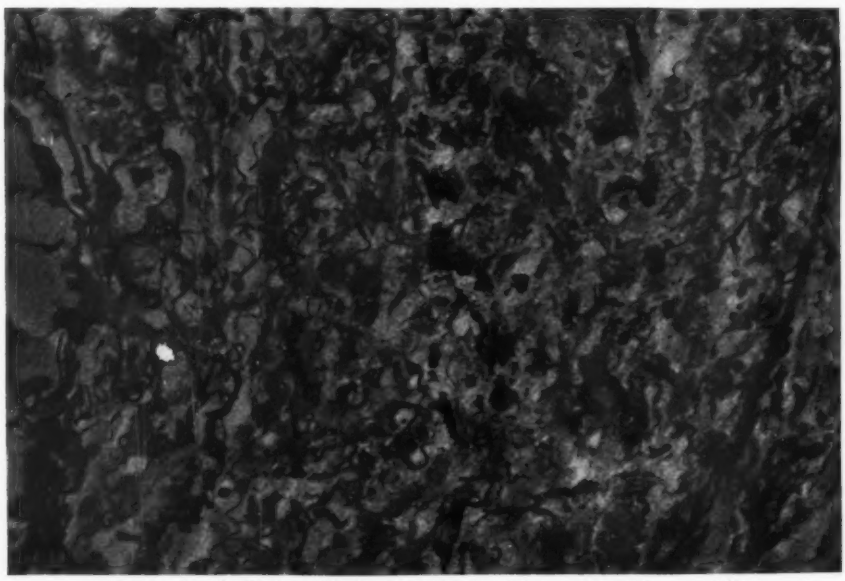

FIGURE 5.—Reticulum fibrils associated with tumor cells. × 400.

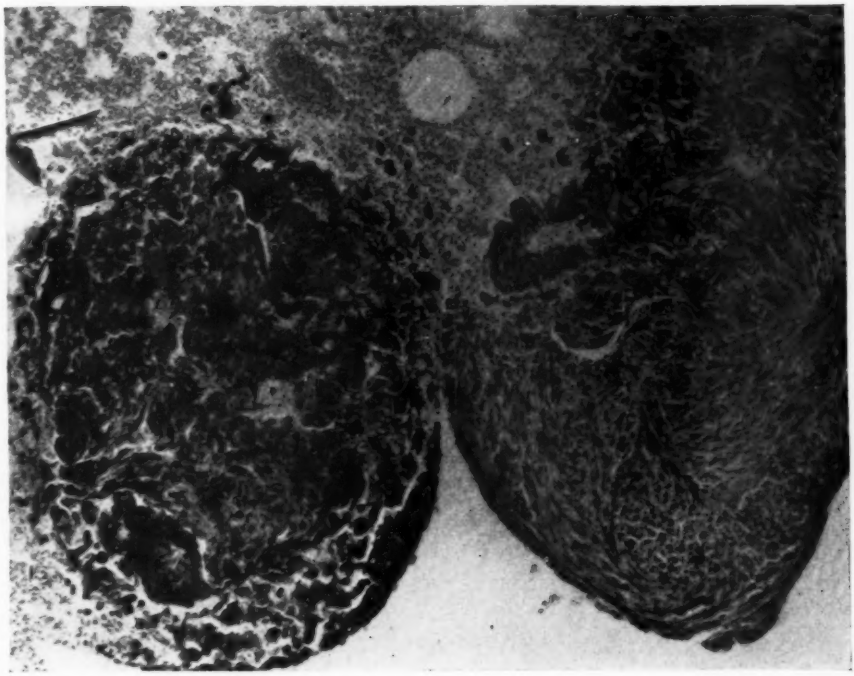

FIGURE 6.—Lung metastases in strain C mouse: One pigmented, the other relatively unpigmented. × 130.

not completely destroyed, which allowed for identification of the tumor in such areas as the pulmonary alveolus and the glomerular tuft of the kidney (fig. 7). The smaller metastases involving the adrenal were usually in the medulla (fig. 8). Lymphatic involvement by tumor was in the subcutaneous tissues around a transplant, in which a perineural lymphatic was distended by neoplasm (fig. 9). In the lung, peribronchial lymphatics were observed plugged with tumor cells. In one strain C mouse that was carrying a third-generation transplant in this strain, the right orbit was distended by a black purply to black mass which protruded outward. Microscopic examination showed this to be a malignant melanoma which lay beside the Harderian gland. The only intrahepatic growth was found in a tumor in which the transplant had been made into the peritoneal cavity.

DISCUSSION

That the melanoma is malignant in both pigmented and albino strains of mice is indicated by the fact that metastases were obtained, and that the metastatic growths were transplantable. The evidence from these pathologic studies is that the tumor has essentially the same characteristics in the albino as in the strain of origin. The tendency to metastasis formation in the S91 melanoma is in distinct contrast with the behavior of the Harding-Passey melanoma, in which, according to the authors (*6*), metastasis occurred on only two occasions in their 4½-year study of the tumor, and even these were questionable. Sugiura (*9*) in a further recent study of the Harding-Passey

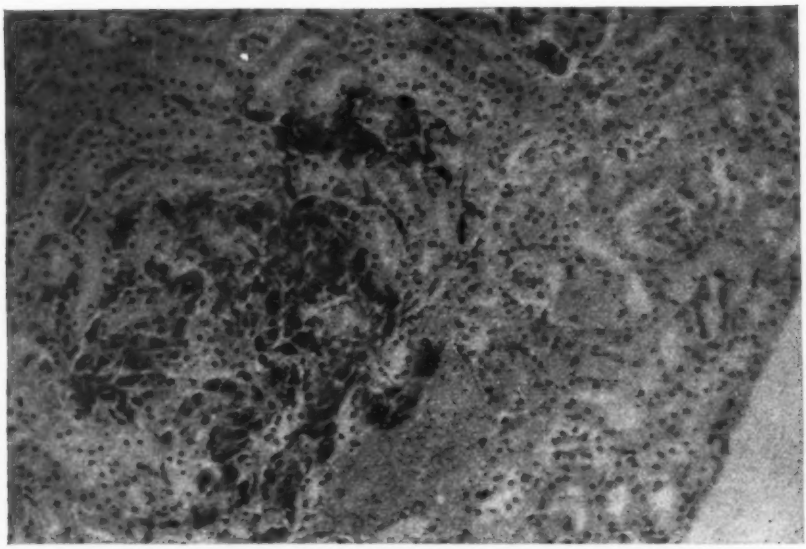

FIGURE 7.—Kidney metastases involving the glomerular tuft. × 210.

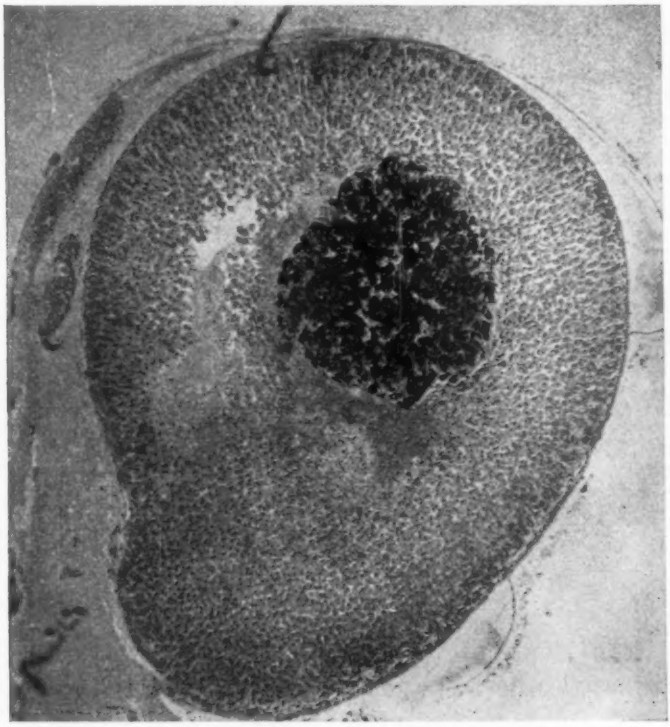

FIGURE 8.—Adrenal metastases involving the medulla. × 50.

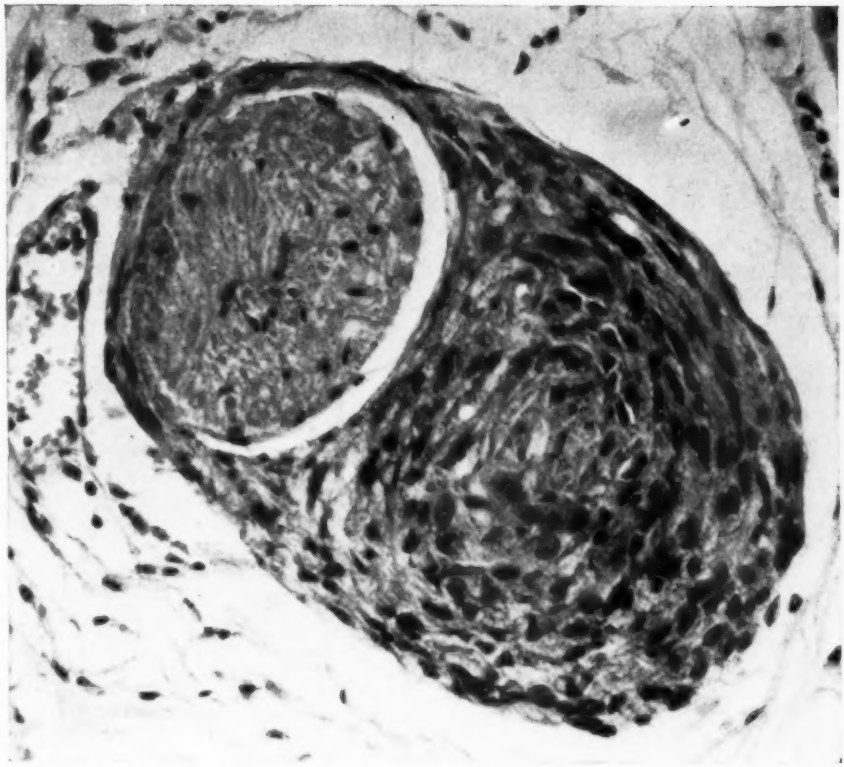

FIGURE 9.—Involvement of perineural lymphatic by neoplasm. × 400.

melanoma found no transplantable metastases but much phagocyted melanotic pigment in liver, spleen, and axillary nodes.

Whether these tumors are epithelial or mesodermal in origin is controversial. The structure of the tumor, with the cells showing numerous fibrils, and the intimate association of reticulum with the individual tumor cell would suggest a mesodermal origin; but whether these criteria are to be taken as absolute indications of a mesothelial origin to the neoplasm is impossible to say.

While some cells are pigmented and others are not, save for the matter of pigment, these two cell types appear structurally alike and may be either closely intermingled or segregated into groups. It has been possible by deliberate selection from the paler portions of the tumors to obtain an almost pigment free strain of the melanoma, although none have been obtained which are completely amelanotic when examined microscopically. Harding and Passey (*6*) reported similar results in their mouse melanoma which arose in a stock chocolate-brown mouse.

Spangler, Murray, and Little made a genetic study of the susceptibility to melanoma S91 of the F_2 albino hybrids of reciprocal A × dba crosses. In their study they assumed a 100-percent nonsusceptibiliy of A strain mice to the tumor. In explanation of their ratio of nonsusceptible to susceptible mice in the hybrid albino line, they put forward a tentative hypothesis. They postulated a gene *M*, which in albino *cc* ani-

mals is necessary for the support of melanin formation, and *m*, its allele. According to this hypothesis, albino animals with the genotype *mmcc* cannot support the growth of S91 melanin tissue. The successful growth of the melanoma in the albino hybrids is explained on the basis of a transfer of the gene *M* from the dba strain into the cross.

While the experiments reported here do not constitute a genetic analysis of the transplantability of this tumor, it may be pointed out that the results do not fall in line with the hypothesis proposed by Spangler, Murray, and Little. In this work, successful transplants were obtained in two different isogenic albino strains. According to the foregoing hypothesis, both of these strains would have to carry the factor *M* supporting melanin formation. While there is no evidence that strain C does not carry this factor, the second albino strain in which the tumor grew was strain A, the strain which they postulated carried the factor *m* and thus did not support melanin formation. It is indicated by these experiments that these melanoma cells, which originated in strain dba and thus can be assumed to carry the full color factor *C*, are capable of continuing to produce pigment regardless of whether they are growing in the environment of a colored mouse or that of an albino mouse.

SUMMARY

Melanoma S91 in the dba strain of origin was successfully transplanted into two isogenic albino strains, A and C. An almost pigment free strain of the tumor was obtained in the strain C mice by deliberate selection of paler portions of the tumors for transfer.

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ROLE OF HEREDITY IN TUMOR DEVELOPMENT

By WALTER E. HESTON, *geneticist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Basic in the study of tumor etiology is a sound understanding of the genetic factors involved. There is convincing evidence that the hereditary elements contained in the fertilized egg have a part in determining whether or not the resultant organism will have a tumor (*1*, *2*). For the average oncologist, however, there exists considerable confusion as to the role of these hereditary factors.

This confusion may be owing in part at least to a lack of clarity in thinking of some of the more basic concepts of genetics, such as the relationship of the gene to the character, the question of dominance, and multiple-factor inheritance. In the following discussion, an attempt is made to develop some of these basic concepts and to point out how they may or do apply to the cancer field and thus to clarify somewhat the position of the genetic factors in carcinogenesis.

GENE-CHARACTER (CANCER) RELATIONSHIP

The most basic concept in genetics is Mendel's law dealing with the segregation of genes. To illustrate this law, let us consider a mouse homozygous for the gene for black coat color, i. e., having the black gene on the loci of both of the homologous chromosomes (*BB*), mated to a mouse homozygous for the gene for brown coat color (*bb*). The F_1 hybrid resulting from this mating receives a gene from each parent and is therefore heterozygous for this pair of factors (*Bb*). Since the action of this combination is comparable with that of the homozygous pair (*BB*), we consider (*B*) as dominant to (*b*), and (*b*) as recessive to (*B*). When these F_1 hybrids are mated, the segregation of these genes in reduction division followed by fertilization results in 1 (*BB*), 2 (*Bb*), and 1 (*bb*), the well-known 3:1 or 1:2:1 ratio of the F_2 generation.

These are the basic steps in all genetic analyses. Genes of all sexually reproducing animals and plants from man down segregate in this manner (the sex-linked genes being excepted). One of the ultimate aims of all genetic analyses is to reduce the problem of the character to terms of the gene, for the genes will behave in this manner regardless of whether they are in a highly inbred population or in a genetically heterogeneous population and regardless of whether they are simple coat-color genes or those influencing cancer development.

Thus, in genetic thinking, while nongenetic factors must be recognized, the emphasis is placed on the gene. The gene is relatively stable, and therefore less confusion arises when definitions are in terms of the gene. A character, rightfully considered as dominant, is affected by a dominant gene or by dominant genes. A genetically homogeneous strain is one in which all animals have the same type of genes. The genic complex for a given character refers to the genes as does the genetic constitution of an animal. A mutation, to the geneticist, usually refers to a gene change. Changes involving the chromosome as a whole are recognized, but these are usually designated as chromosomal mutations or chromosomal aberrations. The gene is basic in Mendelian heredity.

Contrast the foregoing method of illustrating Mendel's law in terms of the gene with the more common but unfortunately the more misleading illustration in terms of the character, i. e., a black mouse mated to a brown mouse gives black offspring, which, when mated together, result in three blacks and one brown. The trouble with this illustration is that it is not always true. It is possible for a black mouse and a brown mouse to have an albino off-

spring, or a dilute brown, or a host of other colors, depending upon other genes which the parents may carry.

While one can predict what gene combinations will emerge from a cross when he knows what genes go into the cross, he cannot predict what characters will emerge from a cross if he merely knows what characters go into the cross. Black-coat-color genes mated to black-coat-color genes result in black-coat-color genes, and specific cancer genes[1] mated to those specific cancer genes result in the specific cancer genes; but just as a black mouse mated to a black mouse may not result in a black mouse, so also we might expect that a cancer mouse mated to a cancer mouse may not give rise to a mouse which will develop a cancer. Aside from the particular gene, the other genes, usually referred to as the genetic background, must be taken into consideration, before one can predict the resultant character.

A second group of factors which must be taken into consideration in predicting the character from the gene is the group of environmental factors. For example, a Himalayan rabbit ($c^{H}c^{H}$), which is a white rabbit with dark extremities, mated to a Himalayan rabbit has offspring of the usual Himalayan pattern not only when the genetic background is suitable but also under suitable temperature conditions. If, at the time the hair is developing, the temperature of the extremities is raised by wrapping them in cloth or by applying xylol, the hair that grows in these areas is white instead of pigmented (*3*). Comparable situations exist in the case of the Siamese cat (*4*) and the albino guinea pig. Bar-eye, vestigial wing, and number of bristles in Drosophila can likewise be altered by changing the temperature (*5*, *6*, *7*); other characters are influenced by nutritional factors (*8*, *9*). In tumor development, environmental factors are particularly important. Breast cancer of the mouse is influenced by the presence or absence of the milk factor, and the so-called induced tumors are influenced by the chemical carcinogen applied.

Genes are inherited; characters are not. The character is the result of the action of the gene, or genes, in a particular environment.

A more complete picture of the gene-character relationship might be developed by visualizing the character as resulting from a chain of events. However, the character under consideration is not necessarily the terminal link (fig. 1).[2] To reiterate, the direction of this chain of events is affected by two sets of factors, inherited factors (for the most part genes)and environmental factors. The genetic factors may be divided into what are referred to as the primary gene or genes and the genetic

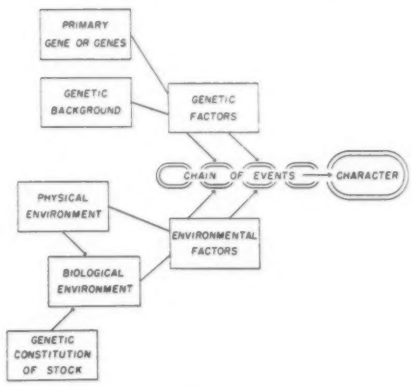

FIGURE 1.

background; the environmental factors may be divided into physical and biologic environment. A part of the biologic environment is the maternal environment, the importance of which has been demonstrated by examples such as the intra-uterine effect on skeletal development, as recently shown by Russell and Green (*13*), the intra-uterine effect on mammary tumors in mice (*14*), and the effect of the milk factor on mammary-tumor development (*15*). This maternal environment may be in-

[1] The term "cancer gene" as used here denotes any gene which can be shown to influence neoplastic development in any way.

[2] For additional information on gene-character relationship, see Wright (*10*, *11*), and Russell (*12*).

fluenced by the genetic constitution of the mother, as was demonstrated recently in the influence of genetic factors on the ability of the female mouse to transmit the milk agent (*16*).

This chain of events may be, and in many cases probably is, a branched chain converging on the character, i. e., there may be several avenues through which the genes may influence the development of the character. There is compelling evidence that mammary-tumor development in the mouse is influenced by genes acting through several different routes. One probable avenue is within the mammary-gland cell itself, but this is not the only one. Recent work (*17*, *18*) suggests that genes may also influence the development of mammary tumors through the hormonal mechanism. In strain A, few virgin females develop mammary tumors although the incidence among the breeders is high, and the virgins give a high incidence after the injection of estrogens. In strain C3H, however, the incidence among virgin as well as breeding females is high. When strain A females are mated to strain C3H males, the introduction of the strain C3H chromatin results in an F_1 generation in which a high tumor incidence occurs among the virgins. If this difference is a result of a difference in hormonal stimulation, then these genes introduced from the strain C3H males are affecting the development of mammary tumors by influencing the hormonal stimulation.

Recent work (*16*) shows that the genetic constitution of the mouse has some influence on the production of the milk factor. Thus this constitutes another avenue, one in which the chain of events carries over from genes in one individual to the character, the mammary tumor, which appears in the young nursed by the individual, and in certain cases even foster-nursed young. It might be pointed out that while the discovery of such extrachromosomal factors at first appears to complicate the picture and to conceal any genetic effect, later these factors are found to provide new routes for attack on the mechanisms through which the genes exert their influence.

IS CANCER INHERITED? HOW IS IT INHERITED?

This general concept of the relation of the gene to the character throws a different light on the somewhat controversial question, Is cancer inherited? As pointed out earlier, strictly speaking we do not inherit characters. We inherit the genes on the chromosomes and the other nuclear and cytoplasmic elements that go to make up the fertilized egg. We do not inherit the cancer. On the other hand, if we choose to define an inherited character as one whose development is influenced by genes according to the concept presented herein, then it would be hard to imagine a cancer whose development is not affected to some extent by genes, just as it is almost impossible to imagine any biologic character whose development is not influenced in some way by the genetic constitution or genes. The question we are asking today is not Is cancer inherited? but How is cancer inherited?

The situation is much simplified when one realizes that by a character most experimental biologists mean a character difference. Oncologists are not so much interested in knowing all the factors that cause A to have a cancer, but they want to know why A has a cancer while B does not. The cause of this difference may be environmental, or it may be genetic, or it may be both, and with homozygous strains it is often not too difficult to determine which it is.

The point is well-illustrated by mammary tumors in mice. The difference between the high tumor incidence of strain A breeding females that were nursed by their own mothers and the low tumor incidence of strain A breeding females that were foster-nursed by C57 black mothers is clearly attributable to a difference in milk factor. It cannot be a genetic difference for both groups have the same genetic constitution. Likewise the difference between

the low tumor incidence of C57 black females nursed by their own mothers and the relatively high tumor incidence of C57 black females foster-nursed by strain A females is also clearly a difference in milk factor. However, the difference between the high tumor incidence of the strain A breeding females nursed by their own strain A mothers and the lower tumor incidence of strain C57 black breeding females foster-nursed by strain A females is a genetic difference. It cannot be a milk-factor influence, for both groups receive the same milk factor. The difference between the high tumor incidence of strain A breeding females and the low tumor incidence of strain A virgin females cannot be a milk-factor difference, or a genetic difference, but it is clearly a hormonal difference. The difference between the high tumor incidence of breeding strain A females nursed by their strain A mothers and the low tumor incidence of the strain C57 black females nursed by their strain C57 black mothers is both a milk-factor and a genetic difference.

Certain investigators are somewhat opposed to referring to the cancer as the character inherited. Many prefer to speak of the susceptibility to cancer development as the inherited character, and in some cases this viewpoint has led to clarification of the thinking on the problem. Considering susceptibility as the character, however, is in part a recognition of the chain of events linking the gene to the character and of merely considering the preceding link, i. e., susceptibility to cancer, instead of the terminal link, the cancer. If and when we describe the links leading to susceptibility, we shall be able to go back link by link to the gene, that which is actually inherited.

Early in cancer research, Von Hansemann, and later Boveri, postulated the hypothesis that a cancer resulted from a change in the chromosomes of the somatic cell, i. e., resulted from a somatic mutation. More recently, many others also have advocated this idea (*19*). Charles and Luce-Clausen (*20*) have demonstrated that the latent period and course of increase in papilloma number among benzene-treated mice were such as could be expected if the tumors were the result of the mutation of a single gene, and Jones (*21*) has shown that growth changes in plants result from somatic chromatin changes. In general, however, observations on cancer development have not upheld the somatic-mutation hypothesis, and at present it must be considered as neither proved nor disproved. It hardly seems essential to assume a chromatin change merely because a cell changes from normal to cancerous. Throughout differentiation, we constantly observe cells changing from one type to another, and it is not generally considered that these cell transformations are immediately preceded by chromatin changes. A more thorough study of normal differentiation would probably throw considerable light on the problem of the change from the normal to the cancer cell.

The gene changes influencing tumor development which are being studied by the geneticist are those observed through segregation in reproduction. As it is known that certain genes may influence mutation rate, it follows that if somatic mutation is instrumental in cancer production, certain of these genes may influence the somatic-mutation rate. It should be emphasized, however, that there is no tangible evidence for this concept as opposed to the evidence that genes do influence tumor production through certain other physiologic avenues.

Almost all our information in answer to the question of how cancer is inherited has been obtained through breeding studies. Some of the early work of this kind was characterized by confusion and misinterpretation, primarily because the work was done with heterozygous stocks, which are unsuitable for the analysis of a character greatly influenced by nongenetic factors. Owing primarily to the foresight and efforts of Dr. C. C. Little and his associates, we now have a large

number of highly inbred strains of mice, in which the expression of cancer can be observed in relation to fixed genotypes.[3] With the use of these inbred strains much progress has been made, whereas without them our present position in cancer research could not have been attained.

Inbreeding is a genetic process, one whereby genotypes characteristic for the different strains are established. Genetic homogeneity is closely approximated following the extensive inbreeding to which each of these strains has been submitted (*23*). Since, in establishing these characteristic gentoypes for each strain, characteristic tumor incidences were also established, it is logical to assume that the tumor incidences are governed to a considerable degree at least by the genotypes. Another explanation, however, is that in the inbreeding process certain "parasites" (*12, p. 347*) were selected, and these caused the characteristic tumor incidence. This situation is probably rare and may in turn be affected by genetic factors, as was pointed out in the case of the milk factor, which may be a virus.

The tumor-incidence data for these inbred strains constitute probably our greatest source of information on tumor inheritance. (See table 1.)

[3] A list of inbred-mouse strains has been published by Snell (*22*).

CANCER NOT A UNIT CHARACTER

One of the most important deductions that can be made from these tumor incidences is that all tumors cannot be grouped as a single character controlled by the same gene or complex of genes. If such were the case, one might expect an inbred strain either to develop all types of tumors or to be resistant to all types. None of these strains develops a high percentage of all types of tumors, and on the other hand, none is completely resistant to all types. One strain may be highly susceptible to one type of tumor and resistant to a second, whereas another strain may be highly susceptible to the second type and resistant to the first. Furthermore, one strain may be highly susceptible to but one type of tumor, whereas a second strain may be susceptible to at least two types.

All these facts point to the necessity of considering the different types of tumors as different characters or different diseases, the inheritance of which probably involves the action of different genes. The picture presented could not be obtained if cancer were inherited as a single trait, unless these variations were the result of environmental variations, and this possibility is highly unlikely. The concept is in accord with the now generally accepted opinion that

TABLE 1.—*Tumor incidence in some of the inbred strains of mice commonly used*

Strain [1]	Mammary-tumor incidence in breeding females (percent)	Lung-tumor incidence (percent)	Leukemia incidence (percent)	Remarks
C3H (*24, 25, 26*)	75 to 100	5 to 10		Hepatomas: females, 10 percent; males, 27 percent.
A (*27, 28, 29, 30, 31*)	70 to 85	80 to 90	Low	
dba (*29, 32, 33, 34*)	55 to 75	Low	Medium (subline 212)	
C57 black (*30, 35, 36*)	<1	<1		
C57 leaden [2] (*31, 37*)	<1	<1		
B alb C [3] (*38*)	<5	20 to 30		
I (*39, 40, 41*)	<1	10 to 20		Develop adenomatous lesions of the stomach.
Swiss (*22, 42*)	19	40 to 50		
C58–(*43*)			90	
Rf (*22, 44*)	Low	32	2	
Ak (*22, 44*)	do	Low	60 to 80	

[1] References to literature are indicated by italic numbers in parentheses.
[2] Also known as strain L and strain M.
[3] Also known as strain C.

cancer is not a single disease but a group of diseases.

These data furthermore give some indication of the manner in which each type of tumor is inherited. As yet none of the mouse tumors have shown clear-cut single-factor inheritance. If a certain type of tumor were inherited as a single-factor character, the gene for tumor being (*T*) with (*t*) as the normal allele, all strains being highly inbred and therefore homozygous would be either (*TT*) or (*tt*). In other words, the strains would fall into two groups, one group developing the tumor with similar incidences, presumably high, and the other being resistant to the tumor. The gradation of incidences displayed by the varous strains, from less than 1 to as high as 90 percent, as is the case for lung tumors, rules out the possibility of single-factor inheritance.

DOMINANCE

This gradation furthermore tends to prohibit the use of the terms "dominant" and "recessive" in respect to tumor inheritance. Slye stressed the recessiveness of cancer when working with her heterogeneous strains. Later, when inbred strains of high susceptibility for certain tumors were produced, it was found that when these strains were outcrossed to resistant strains in many cases the tumors appeared in the F_1 generation, as would be expected with a dominant character. This finding caused a decided change from the recessive viewpoint to that of dominant.

It now appears that the aspect of dominance has been overemphasized. As Little and Gorer (*2*) recently pointed out: "It is unfortunate that the question of dominance has occupied so much attention because at present this must be regarded as a minor issue." It might be stated that at present it is doubtful, in some cases at least, if the term is applicable. The point can be illustrated with the data for lung tumor, which has been referred to as dominant (*30*, *42*). If the tumors of the strain having an incidence of 90 percent were due to dominant genes, what kind of genes give rise to the tumors of the strain having an incidence of only 5 percent? One must assume that some of the genes of the 5-percent strain are the alleles of those of the 90-percent strain and are, therefore, recessive genes. It would thus follow that the tumors of the 5-percent strain are owing, in part at least, to recessive genes. To carry the discussion further, if a tumorous mouse of the 90-percent strain is mated to a resistant mouse, the F_1's develop tumors—the argument for dominance. However, if a tumorous mouse of the 5-percent strain is likewise mated to a resistant mouse, at least most of the F_1's do not develop the tumor, and this is an argument for recessiveness. How would the character behave if the genes were again distributed throughout a heterogeneous population? It is obvious that if we are going to use the terms "dominant" and "recessive" in respect to the genes instead of the character, and this is a more sound way to apply the terms, we had better wait until we have identified those genes.

If one considers susceptibility to the cancer and not the cancer itself, and this is the concept of many, the arguments for dominance are more tenable. Mice of high susceptibility to lung tumors, as judged by the high lung-tumor incidence of the strain, mated to mice of low susceptibility, with a low tumor incidence, give rise to an F_1 generation, most of which develop lung tumors. It should be pointed out, however, that this is not proof that each gene involved is dominant to its respective allele. If the situation involves a physiologic threshold, the tumor appearing when the combined effect of the genetic and nongenetic factors falls above the threshold, it is highly possible that certain of the genes are recessive to their respective alleles or possibly show no dominance. The gradation of the tumor incidence of the different strains supports this concept. This is discussed more fully later.

MULTIPLE-FACTOR ASPECT OF CANCER INHERITANCE

In view of the fact that most of the different types of tumors are obviously not single-factor traits and that the question of dominance is not clear, the question may be asked, What further analysis can be made of the inheritance of these tumors? In other fields of genetics, are there known types of inheritance which might explain such results as are presented here? The answer is in the affirmative, and it lies in the field of multiple factors.[4] This type of inheritance has been investigated extensively in other fields of genetics, especially in plant genetics, but until recently has not been stressed in the cancer field.

Some of the early publications of Loeb (*46*), however, show thinking which was an exception to the general rule at that time. Loeb recognized the quantitative nature of susceptibility to tumor development and pointed out that there must be a quantitative relationship between the hereditary factors and the degree of susceptibility displayed. Since then, scattered evidences of the quantitative aspect have been forthcoming, but for the most part analyses have been of the type more applicable to qualitative characters.

The introduction of the carcinogenic hydrocarbons was instrumental in calling attention to the quantitative nature of susceptibility to tumor development, especially lung tumors. Andervont (*47*) was one of the first to recognize this characteristic of the problem and to refer to susceptibility to induced lung tumors as a matter of degree. It began to appear that susceptibility was probably not a character with definite alternative expression, i. e., we could not consider that animals are either definitely susceptible or definitely resistant, but that all are susceptible to a certain degree. Later, it was found that this degree of susceptibility to induced lung tumors could be measured in two ways: (1) By measuring the latent period; and (2) by counting the nodules in the lung. Both tended to be correlated with spontaneous-tumor incidence. The more susceptible a strain was, as judged by tumor incidence, the shorter the latent period, and the greater the number of nodules that developed. This provided a means for the quantitative analysis of the inheritance of the character.

Quantitative inheritance has been known for a long time in other fields of genetics. As early as 1908 Nilsson-Ehle (see (*45*)), working with kernel color of varying intensities in wheat, and later East (see (*45*)), working with ear length in maize, independently proposed the multiple-factor hypothesis for the inheritance of quantitative characters. This assumes for a given quantitative trait a series of independent genes with cumulative effect.

To illustrate, one might refer to East's experiments. He had two strains of corn, one with length of ear ranging from 5 to 8 cm., and the other from 13 to 21 cm. The F_1 hybrids were intermediate, ranging from 9 to 15 cm. The F_2 hybrids were likewise intermediate, with a range from 7 to 21 cm., which is a wider range than that of the F_1. The variation within the two parent strains and that of the F_1 generation could be attributed to environmental factors, but the increase in variation in the F_2 over that of the F_1 was best considered as the result of segregation of genetic factors since there was no reason to suspect that the environmental factors of the F_2 differed from those of the F_1.

Single-factor inheritance can give an intermediate F_1 if there is incomplete or no dominance. However, a single pair of factors would segregate into but three groups in the F_2, which would be (representing the genes as (*A*) and (*a*)) 1 *AA*, 2 *Aa*, and 1 *aa*. If we increase the number of factors to two, (*A*) and (*B*), with their respective alleles, the number of groups in the F_2 is increased to nine (1 *AABB*, 2 *AABb*, 2*AaBB*, 1 *AAbb*, 4 *AaBb*, 1

[4] For a more completed discussion of multiple-factor inheritance, see Sinnott and Dunn (*45*).

aaBB, 2 *Aabb*, 2*aaBb*, and 1 *aabb*). It is easy to see how, with multiple factors and some nongenetic variation causing an overlapping of the groups, one can get a distribution in the F_2 generation characteristic of that obtained with these quantitative characters.

This original interpretation has been amply confirmed, and more recently analyses of such characters have been carried much further toward the identification of the different factors involved. For example, Lindstrom (*48*) has linked the various factors influencing row number in corn to various qualitative single factors. Castle (*49*) and his co-workers have associated certain qualitative genes in the mouse with the quantitative character size. Brown (*b*), dilution (*d*), and lethal yellow (A^y) increase body size, whereas pink-eye (*p*), leaden (*ln*), and pallid (*pa*) diminish it.

The results of the work on the inheritance of induced pulmonary tumors have fallen in line with those of other experiments on quantitative characters (*50*). When degree of susceptibility was measured by either of two methods, i. e., on the basis of latent period or of the number of nodules, the picture obtained was very much like that which East demonstrated 30 years ago. The F_1's were intermediate to the parent strain; the F_2's were also intermediate and showed greater variation than did the F_1's. Thus, while the tumor incidence of the various inbred strains suggested multiple-factor inheritance, these breeding tests demonstrated it.

Just as other workers have demonstrated linkage between their multiple-factor quantitative traits and single-factor qualitative traits, so has it been possible to show an association between various qualitative genes of the mouse and susceptibility to induced pulmonary tumors. Susceptibility has been demonstrated to be associated with the linked genes waved-2 and shaker-2, the flexed-tail gene, and also the lethal-yellow gene (*51*, *52*). This constitutes an approach to the identification of particular genes involved in the development of a particular type of tumor.

Another concept of multiple-factor inheritance more applicable to the tumor-incidence data is that of the multiple-factor character which fluctuates about a physiologic threshold. One of the best-known examples of this type of character is polydactyly in the guinea pig, analyzed by Wright (*53*). One of his inbred strains of guinea pigs bred true for the four-toed, polydactylous condition; others showed the character in various incidences characteristic for each strain, and still other strains bred true to the normal three-toed condition. The explanation was that the genetic constitution of the strain determined the percentage incidence, the individual variability within the strain being ascribable for the most part to variation in nongenetic factors. The presence or absence of the extra toe depended upon whether or not the combination of factors exceeded or fell below the threshold.

In many respects much of the tumor-incidence data for the various tumors in the mouse appear comparable with Wright's data on polydactyly. In the case of polydactyly, however, the character arises before birth, thus limiting somewhat the possibility of the effect of nongenetic factors, whereas tumors arise much later in life and hence greatly increase the time during which nongenetic factors may enter the picture. This aspect stresses the importance of considering the age factor in tumor study. It would seem that if the genetic constitution of an individual places the individual near the physiologic threshold, the chances would be high that early in life the individual would accumulate nongenetic factors which would carry him above the threshold and cause the tumor to appear; but if the individual were deficient in tumor-susceptibility factors, the chances of accumulating enough nongenetic factors to carry him above the threshold early in life would be slight, but these chances would increase

as the individual got older. It seems logical that degree of inherited susceptibility to tumor formation could be measured on the basis of latent period, as was done in the work just described.

Aside from the foregoing contributions, the analyses of these threshold characters emphasize the necessity of caution against simple genetic interpretations from F_1, F_2, and first-backcross data. In crosses between four-toed family D and three-toed family 2, Wright found that practically all the F_1 hybrids had but three toes, but approximately 25 percent of the F_2 hybrids had four toes, as did 50 percent of the $F_1 \times$ family 2 backcross hybrids. These are ratios which are expected in single-factor inheritance. Wright found also that with breeding tests of the backcross segregants such an interpretation broke down, and it was demonstrated that multiple factors were involved. This fact stresses the importance of breeding tests of the F_2 or backcross segregants before explaining the inheritance of tumor formation on simple-factor segregation. Although results simulating those of simple-gene segregation had been obtained for leukemia, MacDowell's breeding tests of backcross segregants (*54*) refuted such an interpretation and indicated the action of multiple factors.

One might ask how this multiple-factor aspect of cancer inheritance affects the position of the geneticist in regard to human cancer. At the present time, from ancestry data it is impossible to give the probabilities that a complex-multiple-factor trait will appear in an individual of a genetically heterogeneous population, such as the average human population. Therefore, with the exception of the few human neoplasms which appear to have a relatively simple inheritance, statements on probabilities will not be readily forthcoming. If, for a given tumor, susceptibility can be separated into the various physiologic conditions favorable to the development of the tumor, the genetics of certain of these conditions may prove to be more simple. But the value of genetics is not only that of being able to predict 3:1 ratios in the progeny of particular parents. Genetics must be viewed as the study of the physiology and development of the individual in relation to that of his ancestry, the inherited factors serving as the connecting links. Any analysis of the chain of events leading to the appearance of a character—in this case a tumor—will be incomplete in man, as well as in the mouse, without the part played by the genetic factors.

The role of heredity in cancer is complex. Long-time, careful research has established definitely that genetic factors are involved, but the prodigious task now facing the geneticist, reducing the problem to terms of definite genes and then ascertaining the mechanisms through which these genes influence the development of the tumor, is merely begun. However, with the aid of the findings in other fields of cancer research and with the advances made in studying the inheritance of other types of characters, the prospects for future genetic analyses of cancer are encouraging.

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ATTEMPTS TO PRODUCE ANAPHYLAXIS IN GUINEA PIGS WITH A POLYSACCHARIDE FROM *SERRATIA MARCESCENS* (*BACILLUS PRODIGIOSUS*) [1]

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A mixed filtrate of *Bacillus prodigiosus* [3] and the streptococcus of erysipelas has been used for many years in the treatment of lymphosarcoma and Hodgkin's disease (*1, 2, 3*). The toxic effects are very pronounced, thus limiting its use on patients. In connection with further attempts at therapy of tumors with this agent, the possibility that the reactions might be of an anaphylactic nature was studied. The filtrate of *B. prodigiosus* is a polysaccharide, apparently protein-free. Definite hemorrhages into the transplanted sarcoma of mice may be produced by as little as 0.1 cc. of a 1 : 10,000 dilution of a 1-percent solution. This dose, which has been designated as one mouse tumor unit (m. t. u.), contains 0.1γ of nondialyzable solids.

To avoid the use of preservative, a lyophilized preparation of the polysaccharide, designated P-2l, was made. In a few experiments, specifically indicated, a lyophilized preparation, K-8-dl, of the same potency as P-2l but containing a trace of proteinlike material, was employed.

METHOD OF DETERMINING THE MINIMAL LETHAL DOSE

SUBCUTANEOUS INJECTION

The P-2l was taken up in sterile 0.9-percent sodium chloride solution shortly before injection. With concentrations of 1.0 mg. per cubic centimeter, some precipitate could be thrown down on centrifuging; and with lower dilutions, down to 0.1 mg. per cubic centimeter, the fluid was slightly opalescent, and a tiny fraction did not dissolve readily. The injection was made into the anterior abdominal wall of the guinea pig after clipping off the hair and applying an antiseptic, usually 70 percent alcohol. The volume of fluid injected was seldom more than 2 cc. Since fatalities usually occurred within 2 days, an animal living at the end of 7 days is considered to have survived.

INTRACARDIAC INJECTION

The guinea pig was tied down on its back, and the skin over the ventral surface of the chest was prepared as already described. The injection was made through the chest wall, a 27-gage needle being used in most instances. Blood was aspirated about three times during the injection which required 15 to 30 seconds. The volume of fluid injected never exceeded 1.6 cc. and usually was less than 0.5 cc.

METHOD OF DETERMINING ANAPHYLAXIS

Three series of experiments were run using various sensitizing doses of P-2l and a constant shocking dose for each series. The highest sensitizing dose in any series was 1.0 mg. and the lowest 0.00001 mg. per kilogram body weight. The sensitizing dose was given subcutaneously into the anterior abdominal wall after clipping the hair and applying tincture of iodine in the first series, and 70 percent alcohol in the later series.

The volume of fluid injected was always under 1.0 cc. The syringes and needles were washed repeatedly and boiled and kept separate for each dilution throughout the entire study.

The first and second P-2l series were divided into groups, one animal from each group being injected each day. The solutions were injected into the heart at room temperature, except in

[1] The work was authorized by Dr. Carl Voegtlin, former Chief, National Cancer Institute.
[2] The writer is deeply indebted to Dr. M. J. Shear, of the National Cancer Institute, for providing the polysaccharide, and to Dr. E. A. Doisy, of the St. Louis University School of Medicine, for advice and the use of the departmental facilities.
[3] Dr. Shear and his co-workers purified to a high degree the active component of the filtrate of *Bacillus prodigiosus* (*4–8*).

the third series when they were warmed to 37° C.

The intracardiac injections were made slowly through the chest wall by using a fine needle and tuberculin syringe. The animal was then observed carefully for at least 20 minutes, except in the third series when it was observed for 10 minutes only, and then killed with a blow on the head. The control animals received 0.9-percent sodium chloride solution in place of either the sensitizing or the shocking dose. There were eight control animals in the first, nine in the second, and four in the third series. They were killed and examined at the end of 20, or, in the last series, 10 minutes after injection.

Since anaphylactic shock in the guinea pig is characterized by a very large lung, the volume of the lung was measured by the displacement method. A 50-gm. weight was attached to the trachea by a hook and then dropped into a 100-cc. graduate half full of water. The rise in level of the water gives the lung volume, after the displacement due to the weight is subtracted. Control experiments were carried out on the effects of the water level, an open or closed trachea, and the temperature of the water. The specific gravities of the lungs were calculated from the weights and volumes.

The volumes of the lungs of a group of guinea pigs dying of anaphylactic shock from horse serum were determined, and in a few instances the specific gravity was also determined. Both lung volume and specific gravity were determined for all guinea pigs in the second and third P–2l series.

RESULTS

The minimal lethal dose of P-2l when injected cutaneously is found in table 1. Ten milligrams per kilogram was uniformly fatal. Five milligrams per kilogram was 50-percent fatal and may be designated as the L. D. 50. If, after centrifuging, the supernatant fluid was injected, the toxicity was less marked, indicating that at the concentration used (1.25 mg. per 1 cc.) the material was not fully dissolved. Less than half of the guinea pigs died from doses of 3.0 mg. per kilogram, but even with doses of 1.0 mg. per kilogram death frequently occurred (in 25 percent of the animals) possibly indicating variability in tolerance to the toxin.

TABLE 1.—*Minimal fatal dose of polysaccharide P–2l injected into guinea pigs*

Method of injection	Dose per kilogram of body weight	Number of animals	Deaths	Survivals
	Milligrams		*Number*	*Number*
Subcutaneous	10	9	9	0
	5	7	4	3
	[1] 5	3	1	2
	3	9	4	5
	1	12	3	9
	.1	17	1	16
Intracardiac	12	1	1	0
	5	6	5	1
	[2] 5	4	2	2
	1–2.2	5	3	2
	.3	4	1	3
	.1	7	0	7

[1] Centrifuged, injected supernatant fluid.
[2] Centrifuged supernatant fluid used.

The results of the intracardiac injection of P–2l, also given in table 1, indicate that L. D. 50 is in the neighborhood of 1 to 2.2 mg. per kilogram. Five milligrams per kilogram proved fatal in 83 percent of the trials; and even when the material was centrifuged, half of the animals died. Death usually came within 20 hours after the larger doses and within 40 hours after the smaller doses, the toxic effects requiring a period of hours in which to develop.

ANAPHYLAXIS

Of 24 guinea pigs injected with P-2l, none died of anaphylactic shock and none gave a typical picture of this condition. Ten of the animals (table 2, series 1) are listed as doubtful; these showed 1 sign or more than did the control animal, but their behavior was not considered an adequate basis for the diagnosis of anaphylactic shock.

TABLE 2.—*Reaction of guinea pigs to injection of polysaccharide P–2l*

Number of guinea pigs	Sensitizing dose per kilogram body weight	Shocking dose per kilogram body weight [1]	Interval	Animals dead of anaphylactic shock	Probable anaphylactic shock	Animals showing abnormal reactions [2]	Animals with negative anaphylactic shock
Series 1:	*Mg.*	*Mg.*	*Days*	*Number*	*Number*	*Number*	*Number*
4	0.1	0.1	21	0	0	1	3
4	.01	.1	21	0	0	1	3
5	.001	.1	21	0	0	2	3
8	.0001	.1	21	0	0	4	4
3	.00001	.1	21	0	0	2	1
24				0	0	10	14
Series 2:							
8	.1	.3	21–28	0	0	3	5
8	.01	.3	21–28	0	0	3	5
8	.001	.3	21–28	0	0	1	7
8	.0001	.3	21–28	0	0	2	6
32				0	0	9	23
Series 3:							
8	1.0	.1	26	0	0	1	7

[1] In all 3 series, the shocking dose was intracardiac.
[2] Animals classified as showing abnormal reactions did not exhibit all the reactions characteristic of anaphylaxis, and the reactions were relatively mild.

The second series (table 2) was carried out under conditions believed to be more favorable for the elicitation of anaphylactic shock; but none was seen, although nine cases were classified as doubtful because of clinical or necropsy findings.

The third series (table 2) also gave negative results in seven of eight animals; one was doubtful.

None of the control animals developed anaphylactic shock.

The lung volumes of the control guinea pigs were usually between 1.0 and 2.0 cc. per 100 gm. body weight but might be as high as 2.9 or as low as 0.7 cc. (fig. 1). The volume was relatively higher for small guinea pigs. In fatal anaphylactic shock caused by horse serum, the lung volume per 100 gm. of body weight was usually between 3.0 and 4.0 cc. (fig. 2). The controls for this particular series are given. The corresponding volumes for nonfatal anaphylactic shock were 2.2, 2.4, and 2.6 cc., higher than for the usual control animal but within the control range. The specific gravity of the lungs of animals in fatal anaphylactic shock caused by horse serum was

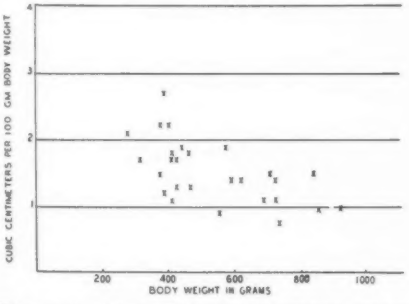

FIGURE 1.—Lung volumes of control guinea pigs (X) receiving saline in place of either the sensitizing or the shocking dose of horse serum.

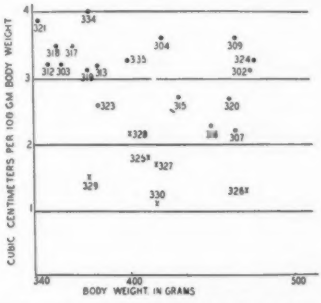

FIGURE 2.—Lung volumes of guinea pigs with anaphylactic shock caused by horse serum, and their controls. ●=Fatal anaphylactic shock; ○=Nonfatal anaphylactic shock; X=Controls.

under 0.4 in 3 animals (table 3), while records on 13 controls ranged from 0.38 to 0.82, the average being 0.51.

TABLE 3.—*Effect on lung of guinea pig of horse serum inducing fatal anaphylactic shock* [1]

Guinea pig No.	Time until respiration stopped	Time until necropsy	Volume of lung	Volume in cubic centimeters/100 gm. body weight	Weight of lung	Specific gravity of lung
	Minutes	*Minutes*	*Cc.*		*Grams*	
350	4.0	20?	15.0	4.1	5.0	0.33
351	4.5	30	12.5	3.3	4.3	.34
352	3.0	12–15	12.0	3.0	4.6	.38

[1] 0.01 cc. horse serum sensitizing dose administered subcutaneously, and about 0.5 cc. horse serum shocking dose (intracardiac); necropsy delayed 12 to 30 minutes after the shocknig dose.

The lung volume in cubic centimeters per 100 gm. body weight in the second P-21 series ranged from 1.1 to 3.0 cc., the average being 1.9 (fig. 3). The controls

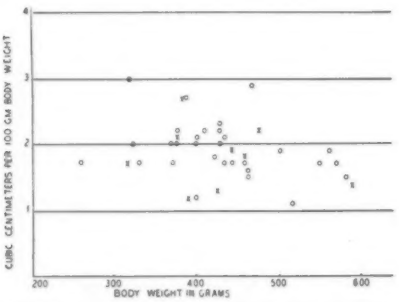

FIGURE 3.—Lung volume of guinea pigs after an attempt to induce shock with P-21, as compared with the controls. O=P-21; X=Controls.

ranged from 1.2 to 2.7, the average being 1.8 cc. Thus, the range and average of the P-21-injected animals was somewhat higher than the values for the controls but considerably below those for animals dying of anaphylactic shock ascribable to horse serum. Here the range was 2.7 to 4.3 for 18 animals, the average being 3.4 cc. The 3 nonfatal instances of anaphylaxis resulting from horse serum varied from 2.2 to 2.6, the average being 2.4 cc.

The specific gravity of the lungs in the second P-21 series was the same as for the controls, namely, 0.53. In only 3 of 41 animals did the specific gravity fall below 0.4. One of the three was a control animal.

In the third P-21 series, both the average lung volume per 100 gm. body weight and the average specific gravity were less than comparable values for the controls. No fatalities resulted from a second injection of the purified filtrate into 13 animals (table 4), although the amount given was approximately the minimal fatal dose in the case of those given the intracardiac injections and was sufficiently large so that some deaths might be expected if such a dose were given subcutaneously for the first time.

TABLE 4.—*Effect of repeated injections on survival of 13 guinea pigs*

Method of administration	Injection per kilogram body weight		Interval between injections	Survival [1]
	First	Second		
	Milligrams	*Milligrams*	*Days*	*Days*
A [2]	0.1	1.0	21	100
	.1	1.0	37	100
	.1	1.0	38	100
	.16	1.0	53	100
B [3]	.3	1.0	105	10
	.3	1.0	105	10
	.3	1.0	105	10
	[4] 5.0	1.0	73	10
	[4] 5.0	1.0	73	10
C [3]	.1	1.0	170	10
	1.0	1.0	34	10
	3.0	1.0	151	10
	5.0	1.0	46	10

[1] All animals alive at time indicated.
[2] 2 injections of P-21 into the heart.
[3] P-21 into the heart at first injection; K-8-dl administered subcutaneously at second injection.
[4] Centrifuged supernatant fluid injected.

DISCUSSION

Before the study on anaphylaxis could be carried out, it was necessary to determine the minimal fatal dose of P-21 when injected under the skin or into the circulation. The subcutaneous dose required, 5.0 mg. per kilogram, was larger than the intracardiac, 1.0-2.2 mg. per kilogram. These doses apply particularly to small guinea pigs.

The largest dose given subcutaneously for sensitization was 1.0 mg. per

kilogram, which is one-fifth the minimal fatal dose. The shocking dose was either 0.1 or 0.3 mg. per kilogram into the heart. The latter dose sometimes caused death, but no effects were observed in the first 20 minutes following the injection, the period of observation for anaphylaxis.

None of the 64 guinea pigs injected died of anaphylactic shock or gave a typical picture of the advanced stages of nonfatal anaphylactic shock. In the 3 series, 20 animals were graded as doubtful. The animals coughed or sneezed 3 times as often as did the controls. An increased depth of respiration, never gasping or extreme, was observed in 4 animals. None of the guinea pigs developed convulsions, and their posture and movements were usually normal. Defecation and urination were seldom observed after the shocking dose. A large lung, found at necropsy without any of the usual anaphylactic phenomena preceding death from a blow, is listed as an abnormal reaction.

The data on lung volume and specific gravity do not offer much support for the view that P–21 produces anaphylaxis.

The effects of repeated injections indicate tolerance rather than hypersensitivity (*9*).

While these negative results do not rule out the possibility that anaphylactic reactions may occur, particularly as some doubtful reactions were obtained, the likelihood of anaphylaxis seems remote.

SUMMARY

The lethal dose (L. D. 50) of P-21, a lyophilized preparation of the polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*) when injected subcutaneously into guinea pigs was found to be 5.0 mg. per kilogram, and 1.0-2.2 mg. per kilogram when injected into the heart.

While some doubtful reactions were obtained, anaphylaxis was not clearly demonstrated in any of the 64 guinea pigs injected with a sensitizing and with a shocking dose.

Some evidence for tolerance to P-21 was obtained.

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EFFECTS OF SUBLETHAL DOSES OF A POLYSACCHARIDE FROM *SERRATIA MARCESCENS* (*BACILLUS PRODIGIOSUS*) ON THE ELECTROCARDIOGRAM, BLOOD ASCORBIC ACID, AND NON-PROTEIN NITROGEN OF THE DOG[1]

By F. E. FRANKE, *assistant professor of physiology*, and DAN RICHERT, *graduate fellow*, *Department of Biochemistry, St. Louis University School of Medicine*

Marked disturbance in the circulation was observed in the dog following the injection of a polysaccharide[2] obtained from *Serratia marcescens* (*Bacillus prodigiosus*). An electrocardiographic study seemed to be indicated. This was combined with a study of the ascorbic acid content of the blood. The production of hemorrhage in transplanted tumors of mice by bacterial filtrates may be accompanied by a considerable reduction of the ascorbic acid content of the tumor (*1*, *2*), and the injection of ascorbic acid may prevent the appearance of such a hemorrhage and subsequent regression of the tumor (*3*). However, later work did not support this view (*4*).

EXPERIMENTAL PROCEDURE

A polysccharide was isolated from the *Bacillus prodigiosus* filtrate, and its properties were described by Hartwell, Shear, and Adams (*4*).

The electrocardiograms were obtained with the dog lying on its back, the three standard leads being used. The skin was shaved at the site of application of the electrodes. The pad of cotton between the electrode and skin and the bandage holding the electrodes in place were soaked in a saturated saline solution. The electrocardiograms were taken with a two-stage-resistance capacity coupled amplifier and a Sanborn electrocardiograph galavanometer. The time constant of the amplifier coupling circuit was sufficiently long (3.0 seconds) to produce no distortion, as evidenced by a check of the instrument against a string galvanometer. Time lines were recorded at 0.05-second intervals by means of an electrically driven synchronous motor timer. Three or four control records were obtained on each animal, usually days apart. With the exception of one animal, three or four electrocardiograms were obtained from each dog after the injection of the polysaccharide, the usual interval between records being about 6 hours.

The ascorbic acid and nonprotein nitrogen of the blood were determined in six dogs (three male, three female) before and after the injection of the polysaccharide. The weight of the dogs ranged from 8 to 12 kg. No anesthetic was used at any time. The blood sample was usually obtained from the saphenous vein, with aseptic precautions, but in a few instances it was obtained from the femoral vein or from the heart after death.

The volume of the blood sample was about 12 cc., which was sufficient for duplicate determinations. Three control samples were taken at 5-to-6-hour intervals for the ascorbic acid determinations, but in three animals only one control observation of nonprotein nitrogen was made. With one exception, at least three blood samples were taken after the injection of the polysaccharide. The interval between the post-injection samples was usually about 6 hours but varied from 4 to 12 hours. Ascorbic acid was determined by the method of Roe and Kuether (*5*) and the nonprotein nitrogen by applying the photelometric method of Hoffman and Osgood (*6*) to the tungstic acid filtrates.

The urinary excretion of ascorbic acid was studied in four dogs. The bladder was emptied by catheterization at the beginning and at the end of each period of observation. The dogs

[1] The work reported herein was authorized by Dr. Carl Voegtlin, formerly Chief, National Cancer Institute.

[2] The polysaccharide used in this investigation was supplied by Dr. M. J. Shear, of the National Cancer Institute.

were kept in metabolism cages throughout the experiment, except for temporary removal for examination. Since the polysaccharide caused vomiting and diarrhea, some urine samples were contaminated as indicated later.

Convulsions sometimes preceded the death of the unanesthetized animal injected with the polysaccharide. This effect suggested the possibility of tetany. The serum calcium was determined in two additional dogs, which were medium-sized, one a male, the other a female. Wang's method (7) was used for the serum-calcium determinations.

A film of a saturated oxalate solution was present in the syringes used for collecting all blood samples except those used in the blood calcium study.

The polysaccharide was fatal to two of the dogs (Nos. 501 and 505). The other four dogs of this series were killed between the twentieth and the thirty-first hour after injection of the active principle, by inserting needle electrodes through the chest wall into the heart and producing ventricular fibrillation with a tetanizing current. All dogs were examined at necropsy, usually immediately after death.

RESULTS

The electrocardiographic results are summarized in table 1. The heart rate increased following the injection of the polysaccharide. The average rate in the control series was 107, with a range of 66 to 160 per minute, and the post-injection average was 145, with a range of 102 to 190 per minute. Inverted P waves were seen in 3 of the control records, each in a different animal. Upright, inverted, and diphasic T waves were found in all leads.

A reversal of the T wave from upright to inverted occurred four times in lead 1, and a change from inverted to upright was found once in lead 2 and once in lead 3. The reversals were seen only after the injection of the active principle. Changes in the direction of the T wave from upward or downward to diphasic were occasionally seen in all leads.

The P-R interval and the duration of the QRS complex were similar in the control and the injected series and were not abnormally prolonged in any case. The duration of the electrical systole, the Q-T interval, decreased with the more rapid heart rate from an average of 0.208 to 0.183 second. The largest Q waves were seen in lead 1, both before and after the injection of the polysaccharide, but large Q waves were more frequently present in lead 2 than in the other leads. The incidence of the S wave, when all records are included, was highest in lead 3 and lowest in lead 1. The incidence of the S wave decreased in lead 1 and increased in lead 3 after the active principle was administered.

The RS-T segment was not raised or lowered as much as 1 mm. except in two cycles of one record. Accompanying this rise in the level of the RS-T segment, which amounted to about 3 mm., there was an increased amplitude of the R wave and a change in the contour of the preceding P wave. The P-R and QRS intervals were normal in all cycles of this lead (3) taken 21 hours after the injection of the polysaccharide.

No premature beats were recorded. In one instance, in a dog with a very pronounced respiratory arrhythmia, there was a slightly longer pause after the injection than in the control records.

The effect of the polysaccharide on the concentration of the ascorbic acid in the blood is shown in table 2. The maximal rises in blood ascorbic acid over the average values of the controls were 68, 75, 109, and 130 percent in dogs 506, 505, 504, and 501, respectively. The maximal decrease in blood ascorbic acid in dogs 502 and 503 was 46 and 64 percent, respectively. The concentration of the ascorbic acid in the blood following the injection of the polysaccharide increased or decreased.

TABLE 1.—*Effect of the polysaccharide on the electrocardiogram of the dog*

Direction of the waves						Average amplitude of the waves in millimeters						Range of variation of the waves in millimeters					
Controls			After injection			Controls			After injection			Controls			After injection		
L_1	L_2	L_3	L_1	L_2	L_3	L_1	L_2	L_3	L_1	L_2	L_3	L_1	L_2	L_3	L_1	L_2	L_3
P WAVES																	
12u	16u	16u	16u	17u	17u	0.7	1.5	1.3	0.7	2.2	2.0	0.4–1.1	0.7–2.3	0.7–2.7	0.3–1.0	1.0–3.5	0.5–3.0
3d						.9						.9–1.0					
1is			1is														
Q WAVES																	
4d	13d	9d	9d	10d	3d	1.4	0.8	0.7	1.1	1.0	1.5	0.5–3.5	0.5–2.0	0.5–1.4	0.5–2.4	0.4–2.3	0.5–3.5
12is	3is	8is	8is	7is	14is												
R WAVES																	
14u	16u	16u	17u	17u	17u	2.8	13.1	11.1	2.8	13.0	10.9	0.9–5.0	8.3–19.0	0.4–15.2	1.0–4.3	8.0–21.0	4.0–21.0
2is																	
S WAVES																	
3d	6d	4d	3d	2d	7d	1.9	1.7	0.8	0.8	1.3	1.0	0.7–2.5	0.3–3.0	0.5–1.5	0.5–1.0	1.0–1.5	0.5–1.5
13is	10is	12is	14is	15is	10is												
T WAVES																	
12u	9u	8u	7u	10u	11u	1.5	1.9	1.9	2.1	1.8	1.6	0.3–4.0 up	0.7–3.5 up	0.7–4.3 up	0.5–4.3 up	0.5–3.2 up	0.5–3.2 up
	1dp	4dp		1dp	2dp		.5	.6		1.0	.8		.5dp	.4–1.0 dp		1.0dp	.5–1.0 dp
	6d	4d	5d	4d	3d		1.9	2.0	.7	2.5	2.2		.4–3.0 d	1.8–2.0 d	.5–.9 d	2.0–4.0 d	2.0–2.5 d
4is			5is	2is	1is												

	Average duration in seconds						Duration—Range of variation					
QRS complex	0.04	0.04	0.04	0.04	0.04	0.04	0.04–0.05	0.03–0.06	0.04–0.05	0.03–0.05	0–03–0.05	0.04–0.05
QRS–T	.21	.21	.21	.17	.19	.19	.19–.24	.10–.25	.19–.22	.15–.19	.18–.20	.18–.20
P–R interval	.10	.10	.11	.10	.10	.10	.07–.12	.0–.13	.09–.12	.08–.12	.08–.13	.07–.15

L_1, L_2, and L_3 represent leads 1, 2, and 3, respectively. "u" signifies up; "d" down; "dp" diphasic; "is" isolectric or wave absent.

In every animal the disturbance was considerably beyond the range of its control determinations.

TABLE 2.—*Effect of a polysaccharide from* Bacillus prodigiosus *on the blood ascorbic acid of dogs*

Dog No.	Polysaccharide injected per kilogram body weight	Ascorbic acid in—			
		Control dogs		Treated dogs	
		Range	Average	Range	Average
	Milligrams	*Mg. pct.*	*Mg. pct.*	*Mg. pct.*	*Mg.pct.*
501	[1] 5.0	0.65–0.88	0.81	1.86	1.86
502	[1] 3.0	.76– .88	.84	.65– .45	.55
503	[1] 5.7	.37– .67	.56	.54– .20	.34
504	[1] 5.0	.87–1.05	.93	.87–1.94	1.28
505	[2] 3.9	.60–1.00	.83	.80–1.45	.98
506	[2] 5.7	.90–1.10	1.04	1.25–1.75	1.51

[1] Injected subcutaneously.
[2] Injected intraperitoneally.

Following the injection of the polysaccharide, the rate of urinary excretion of ascorbic acid was unchanged in dog 505, rose 145 percent in 16 hours in dog 506, and showed an initial rise and a late fall in the other two dogs. In dog 503 a rise of 25 percent in the first 17 hours was followed by a decrease of 61 percent in the next 12.5 hours. In dog 504 a rise of 144 percent in the first 11 hours was followed by a fall of 52 percent in the next 12 hours. The control determinations ranged from 0.96 to 1.41 mg. per hour, and the period of urine collection varied from 12 to 40 hours. Contamination of the urine with vomitus and feces occurred after the injection of the polysaccharide in samples from dogs 505 and 506 and the last sample from dog 504.

Three of the dogs showed a progressive rise in the nonprotein nitrogen of the blood (table 3), although polyuria was present at the time. Two dogs showed little changes. The urinary nitrogen was not determined.

The serum calcium was determined in two dogs. In each dog, two determinations were made before and two after the injection of a lethal dose of the polysaccharide. The difference between the average control and the average post-injection determination was 0.1 mg. percent for one dog, and 0.2 mg. percent for the other. All determinations fell between 8.3 and 10.7 mg. percent.

TABLE 3.—*Effect of a polysaccharide from* Bacillus prodigiosus *on the blood nonprotein nitrogen of dogs*

Dog No.	Dose of polysaccharide injected per kilogram body weight	Nonprotein nitrogen in—			
		Controls		Treated dogs	
		Range	Average	Range	Average
	Milligrams	*Mg. pct.*	*Mg. pct.*	*Mg. pct.*	*Mg. pct.*
501	[1] 5.0	[2] 30	30	44	44
502	[1] 3.0	[2] 33.2	33.2	30.2–32	31.2
503	[1] 5.7	[2] 42.1	42.1	41 –48	44.4
504	[1] 5.0	26.5–30.0	28.7	43 –75	61.7
505	[3] 3.9	33.5–40.0	36.1	37 –80	54.4
506	[3] 5.7	32.0–33.5	32.5	40 –68	55.3

[1] Injected subcutaneously.
[2] 1 control determination.
[3] Injected intraperitoneally.

DISCUSSION

There were no ectopic beats and no disturbance in atrioventricular conduction. The respiratory arrhythmia was less pronounced with the faster heart rates following the injection of the polysaccharide.

Inverted P waves have been reported in normal, unanesthetized dogs (*8*, *9*), and they were seen only in our control series. The T waves of normal unanesthetized dogs are particularly likely to vary in direction and amplitude (*8*, *9*, *10*). Negative T waves are frequently found in any of the three standard leads. Lalich (*8*) observed reversal in the direction of the T wave in 19 of 24 dogs. Reversal of the T wave was observed in 4 of our 5 animals, a total of 6 reversals being noted, 3 in lead 1, 1 in lead 2, and 2 in lead 3. All were in injected animals. Since the incidence is less than that observed by Lalich in normal animals, it probably has no clinical significance.

The electrocardiograms were essentially negative except for the cardiac acceleration, a sinus tachycardia.

Evidence of toxicity, such as fever and tachycardia, was observed in all the dogs following injection of the polysaccharide. The concentration of ascorbic acid in the blood decreased in the two dogs showing the mildest toxic effects, the least change in blood nonprotein nitrogen and little or no congestion and hemorrhage of the gastrointestinal tract at necropsy.

The other four dogs showed a noticeable rise in the ascorbic acid level of the blood, a rise in nonprotein nitrogen, which was usually progressive, and moderate to extensive gastrointestinal congestion and hemorrhage were found at necropsy.

In ascorbic acid deficiency as seen in scurvy, the hemorrhages are associated with a low concentration of ascorbic acid in the blood. But the possibility exists that a temporary high level of blood ascorbic acid might be owing to a rapid depletion of the tissues, the ascorbic acid passing into the blood stream. We have some evidence of increased urinary excretion. This was observed in dogs not taking food and suggests increased mobilization or increased synthesis. But it would seem unlikely that depletion of ascorbic acid in the capillary wall sufficient to result in hemorrhages would occur at a time when there is an increase in the concentration of this highly diffusible vitamin in the blood.

Sufficient information is not available to determine the mechanism of this progressive rise in nonprotein nitrogen.

SUMMARY

No significant changes, except the development of a sinus tachycardia, were observed in the electrocardiograms of dogs that had received sublethal doses of a polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*).

The effect on the ascorbic acid concentration of the blood was variable, a rise being observed more frequently than a fall.

The blood nonprotein nitrogen usually rose.

Significant changes in serum calcium were not observed.

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ACTION OF TOXIC DOSES OF THE POLYSACCHARIDE FROM *SERRATIA MARCESCENS* (*BACILLUS PRODIGIOSUS*) ON THE DOG AND GUINEA PIG

By F. E. FRANKE, *assistant professor of physiology, St. Louis University School of Medicine*

The polysaccharide[1] isolated from the culture filtrate of *Serratia marcescens* (*Bacillus prodigiosus*) by Shear and Turner (*1*) was studied for its toxic properties. The anaphylactic studies on the guinea pig, and the effects on the electrocardiogram, blood ascorbic acid, and nonprotein nitrogen obtained with sublethal doses are reported in the preceding papers (*2*, *3*).

MATERIALS AND METHOD

Because anesthesia seemed to modify the course of events significantly, 21 unanesthetized dogs were injected with the polysaccharide, and changes in heart rate, respiration, rectal temperature, and in certain reflexes, such as the knee jerk, the corneal reflex, the flexion reflex to noxious stimuli, and the righting reflex were routinely observed. The changes in hematocrit determinations and hemoglobin concentration were followed in 5 dogs. The Sahli method was used for the estimation of hemoglobin. The blood samples were placed in Wintrobe tubes and centrifuged at the same speed and for the same length of time as for the hematocrit determinations. The coagulation time was observed in 10 dogs; 1 method was to sprinkle a few drops of blood from a syringe on a porcelain dish and note the time required for a loss of fluidity. The Howell method was used in some experiments. In 3 unanesthetized dogs, the capillary fragility was tested by applying suction through a small cup to symmetrical areas of the skin of the abdomen just craniad to the pubis, before and 3 to 6 hours after injection of the polysaccharide. Negative pressures of 400 to 700 mm. of mercury were used. The arterial blood pressure was determined in a few animals by inserting into the artery a needle connected with a manometer. The animals were usually necropsied soon after death, and the entire length of the gastrointestinal tract was explored. Microscopic sections were made of thoracic and abdominal viscera.

Two types of anesthesia were used: 8.0 mg. of morphine sulfate per kilogram body weight and 1.0 gm. of urethane per kilogram were given intramuscularly at least 2 hours before operating, or 28 mg. of nembutal per kilogram intravenously a few minutes before operating. Twelve dogs were used in this series. The blood-pressure record was obtained by connecting a cannula in the femoral or carotid artery to a mercury manometer or a metal bellows type of manometer writing on a long-roll kymograph. The respirations were recorded with a pneumograph and tambour; the rectal temperatures were taken; and circulatory and respiratory reflexes were observed. Necropsies were made on the animals.

In another experiment, six dogs were treated with adrenalin. Five of them were anesthetized, and observations were made similar to those just described. The adrenalin was injected subcutaneously into one animal, intravenously into two, and three dogs received adrenalin in oil intramuscularly. An attempt was made to prevent the fall in blood pressure owing to the polysaccharide or to restore the blood pressure to a normal level.

To test changes in capillary permeability, 10 pairs of male rats were used. One rat of each pair served as a control and received a single subcutaneous injection of saline. His mate received a single subcutaneous injection of polysaccharide, which was administered in doses of 5 mg. per kilogram, with the exception of 1 rat which received 10 mg. After an interval varying with different pairs from 3 to 6.3 hours, all rats received an intracardiac

[1] Supplied through the courtesy of Dr. M. J. Shear, of the National Cancer Institute.

injection of 1 percent trypan blue solution, 10 cc. per kilogram of body weight. An increase in capillary permeability was indicated by discoloration of the skin and by any staining of the viscera and of the tissues at the site of subcutaneous injection, as seen at necropsy. The animals were usually killed 2 to 9 hours after the dye was injected.

The toxic effects of the polysaccharide on guinea pigs were noted when the minimal lethal dose was determined for subcutaneous and intracardiac injection prior to studies on anaphylaxis. The necropsy findings were usually recorded when fatalities occurred.

RESULTS

EXPERIMENTS WITH DOGS

In the dose range of 1 to 8 mg. per kilogram, the polysaccharide was fatal to 9 of 21 unanesthetized dogs. Seven died between 12 and 28 hours, and 2 in 3 hours after injection. Six dogs recovered. A like number were killed, usually about 24 hours after injection. Two of those killed probably would have died as they were having convulsions, and two were definitely recovering. The dose of polysaccharide and the route of administration are given in table 1. After administration of the polysaccharide, the dog lay down or walked about but appeared quieter, and painful stimuli seemed less disturbing. Retching, vomiting, and diarrhea were frequently seen in the first hour and occurred sooner or later in the great majority of the animals. The stools were macroscopically bloody in 5 animals.

TABLE 1.—*Minimal lethal dose of polysaccharide in unanesthetized dogs*

Method of injection	Dose[1]	Number of dogs	Dogs dead	Dogs recovered	Dogs killed	Percentage of deaths
Subcutaneous	1-4	7	2	4	1	29
	5-8	4	2	------	2	50
Intraperitoneal	4-8	7	5	------	2	71
Intravenous	1-2	3	------	2	1	0
Total	------	21	9	6	6	43

[1] In milligrams per kilogram body weight of animal.

The rectal temperature tended to rise about 2° C. within a few hours; the greatest rise observed was 3.2°. The heart rate accelerated significantly, as shown by statistical analysis of the electrocardiograms. The respirations were usually normal in rate and amplitude, but several dogs showed periods of fast, deep, and even noisy respirations. The changes in rectal temperature, respiratory rate, and heart rate are shown in figure 1. The knee jerk was active and occasionally exaggerated; the flexion reflex became depressed; and the corneal reflex remained until just before death. The dogs refused to eat solid food, but several drank large quantities of water. Drinking was followed by vomiting or diuresis. The changes in weight were determined in 5 animals. All lost weight, the maximal loss for each animal varying from 2.6 to 13 percent of the body weight. The average maximum loss for the group was 8 percent. Hematocrit and hemoglobin determinations gave variable results: a rise, a fall, or no change was observed. The coagulation time was abnormally prolonged at some stage of the poisoning in 3 of 10 dogs. One dog developed a pronounced tendency to bleed, but the clotting time remained normal and within the control range. Two of three dogs showed increased permeability of the skin capillaries, but the differences were not great.

Albumin appeared in the urine of 1 animal after injection, although absent earlier.

In an advanced stage of the poisoning, the arterial blood pressures, as determined by direct measurement in three dogs were 30, 50, and 50–80 mm. mercury. The animals' gait became unsteady, falling was noted, or prostration developed. The animals lay on the side and were unable to stand. Twitching of the ears, of the facial muscles, and of the extremities often preceded the convulsions. The neck and extremities were rigidly extended in tonic convulsions. Occasionally clonic convulsions and running movements were seen. The convulsions immediately

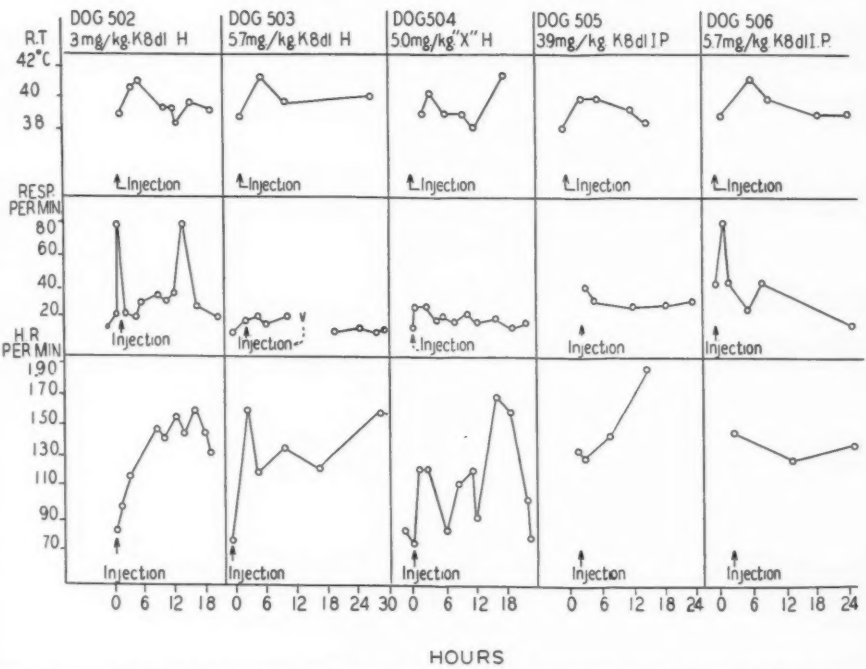

FIGURE 1.—Effect of the polysaccharide on the rectal temperature, respiratory and heart rates of unanesthetized dogs. R. T., rectal temperature; Resp., respiration; H. R., heart rate; H., subcutaneous injection; I. P., intraperitoneal injection.

preceded death or occurred intermittently for over an hour before death.

Eight dogs died in convulsions. Before death, the heart rate became very slow, and cyanosis developed. The pupils dilated widely, and the corneal reflex disappeared just before death. The heart and respiration failed simultaneously in one; while in four the heart continued to beat after the convulsion, although respiration was not resumed.

Eight dogs under morphine-urethane anesthesia received the polysaccharide intravenously in doses ranging from 0.25 to 4.25 mg. per kilogram. Four of the dogs showed a progressive fall in blood pressure, death resulting in 1 to 5 hours (fig. 2). A definite fall in blood pressure usually began in the first half hour, and the blood pressure reached shock level (22–56 mm. mercury) with in 1 to 2 hours. The respiratory rate and amplitude increased after the injection. When the blood pressure reached or remained at a low level for some time, the respirations were inadequate; they became slow, deep, and irregular, then gradually diminished in amplitude. The heart continued to beat regularly, usually for a short period after respirations had stopped. Aspiration of vomitus occurred in one dog after the blood pressure had fallen to 50 mm. mercury and was a factor in its death.

The remaining four dogs under morphine-urethane anesthesia were killed after being observed from 1 to 5 hours. One showed a slight rise in blood pressure, and one a slight fall during a relatively short period of observation (1 to 1.5 hours). The other two showed

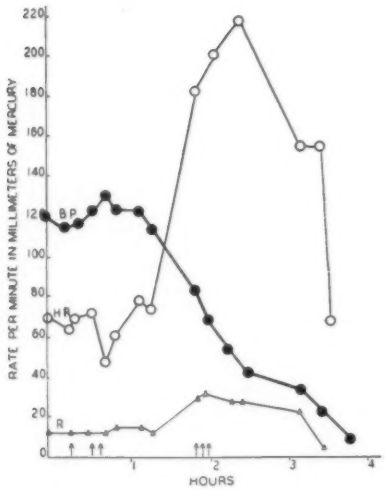

FIGURE 2.—Effect of polysaccharide on arterial blood, pressure, heart rate, and respiration of a dog under morphine-and-urethane anesthesia. B. P., blood pressure; H. R., heart rate; R., respiration. Time in hours. ↑ 15 cc. of Locke's solution intravenously; ↑↑ and ↑↑↑ 0.5 and 0.36 mg. of polysaccharide per kilogram body weight intravenously.

a fall in blood pressure to shock level in 2 hours and an increase in heart rate and respirations.

Traube-Hering waves occurred in the blood-pressure records in two animals in the morphine-urethane series. Stimulation of the central end of a sensory nerve, such as the sciatic or the saphenous, caused the usual increase in rate and depth of respirations and a slight or moderate rise in blood pressure. The right vagus was cut in the neck. Stimulation of the peripheral end caused cessation of heart beat and fall in blood pressure, and stimulation of the central end caused inhibition of respiration. The gastrocnemius muscle responded to sciatic nerve stimulation. Artificial respiration given to one animal whose heart was beating after respiration had failed did not revive the animal, although there was a temporary slight rise in blood pressure. The rectal temperature of the dogs fell in the long experiments under anesthesia.

Four dogs anesthetized with nembutal received the polysaccharide intravenously in doses of 1.25 to 4.2 mg. per kilogram body weight. The blood pressure usually fell rapidly after the injection, fluctuated at a low level (25–75 mm. mercury) for 1 to 3 hours, then recovered partially; in two animals there was almost full recovery of the blood pressure within 5 hours (fig. 3).

The pulse pressure consistently showed definite decreases which were not accounted for by changes in heart rate. The respirations were usually increased in rate or depth, or in both. The heart rate was fast before injection and did not show the acceleration seen in the unanesthetized dogs after administration of the polysaccharide. Stimulation of the central and periph-

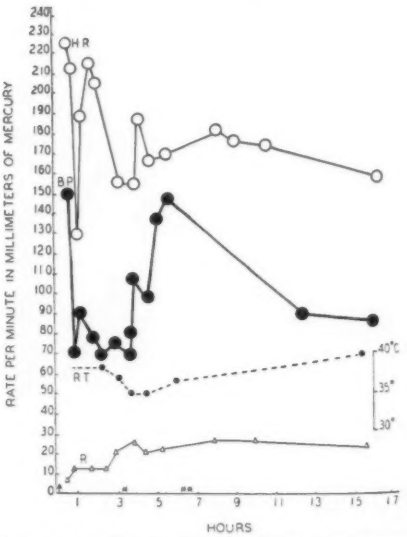

FIGURE 3.—Effect of the polysaccharide on the arterial blood pressure, heart rate, respiration, and rectal temperature of a dog anesthetized with nembutal.

H. R., heart rate; R., respiration; B. P., blood pressure; R. T., rectal temperature. Ordinate for rectal temperature on the right: for the heart rate, respiration, and blood pressure on the left. Time in intervals of 1 hour. 28 mg. nembutal per kilogram body weight intravenously. 4 mg. polysaccharide intravenously in 110 cc. saline at 38° C. *Heat applied. **Heat off.

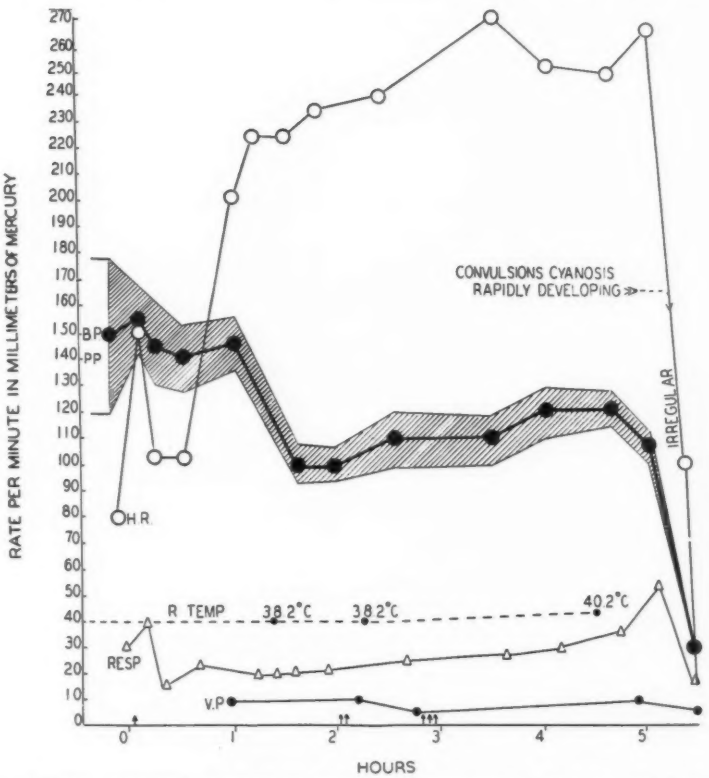

FIGURE 4.—Effects of adrenal therapy on circulation, respiration, and temperature of a dog previously given polysaccharide intravenously.

P. P., pulse pressure; B. P., mean femoral arterial blood pressure; H. R., heart rate; R., respiration; R. T., rectal temperature; V. P., pressure in femoral vein. Time in 1-hour intervals.

↑ 2.5 mg. of polysaccharide per kilogram body weight intravenously. Dog under morphine-and-urethane anesthesia.

↑ ↑ Injection of 0.07 mg. adrenalin in oil, 1:500, per kilogram body weight.

↑ ↑ ↑ Injection of 0.15 mg. adrenalin in oil per kilogram body weight.

eral ends of the vagus produced characteristically normal effects on heart rate, blood pressure, and respiration. Stimulation of the central end of the sciatic nerve markedly increased heart rate and respiration.

The two dogs showing the greatest recovery received some treatment. External heat was applied after the fall in blood pressure, and one dog was given 316 cc. (about 9 percent of the body weight) of saline intravenously. Both of these dogs recovered consciousness and were able to stand and to drink water. They were killed because the operative procedures were not carried out aseptically. One dog, whose blood pressure was taken after the nembutal anesthesia had worn off, showed a late drop in blood pressure.

The anesthetized dogs differed from the unanesthetized by the absence of retching, vomiting, defecation, urination, and convulsions. The rectal temperature tended to fall instead of rise. Anesthetized animals died in a few

hours or began to recover from threatening circulatory failure. The unanesthetized dogs usually did not seem seriously ill until many hours after the injection of the polysaccharide.

The life of a moribund dog was apparently prolonged by artificial respiration and the intravenous administration of adrenalin. Following each injection the blood pressure rose from a very low level to more than 200 mm. mercury; but with repeated injections it soon fell to its former low level and could not be maintained. The intramuscular and hypodermic administration of adrenalin did not prevent the fall in blood pressure or restore it to normal in our small series (fig. 4).

Except for the immediate effects of intravenous injection, the typical response of the animal to adrenalin was a rise in heart rate, a decrease in pulse pressure, an increase in rate of respiration, and a rise in rectal temperature, with convulsions and death in 5 to 9 hours (fig. 4 and table 2).

TABLE 2.—*Adrenalin therapy administered to dogs following injection of the polysaccharide*

Dog No.	Polysaccharide dose per kilogram body weight	Adrenalin		Time from administration of adrenalin until death
		Route [1]	Dose per kilogram body weight	
	Milligrams		*Milligrams*	*Hours*
303 [2]	1.0	I. V.	0.51	([3])
306 [2]	3.7	I. M.[4]	.47	5
1001 [5]	2.2	S.	.54	9
1002 [5]	2.5	I. M.[4]	.22	6
1004 [5]	2.3	I. V.	.53	6
1101 [6]	2.2	I. M.[4]	.15	7

[1] Abbreviations here used: I. V., intravenous; I. M., intramuscular; S., subcutaneous.
[2] Received nembutal anesthesia.
[3] Did not die, but was killed 4 hours after administration of adrenalin therapy.
[4] Adrenalin was administered in oil.
[5] Received morphine and urethane anesthesia.
[6] No anesthesia.

The hemorrhagic effects of polysaccharide were manifest even in normal tissues by bleeding into the intestinal canal and into lymphoid tissue (figs. 5–7). The mucosa of the intestines, particularly the small intestine, was intensely congested and hemorrhagic in

FIGURE 5.—Hemorrhage in the central portion of the lymph follicle of the dog's small intestine, following injection of polysaccharide. × 50.

about one-fourth of the experimental animals. Congestion and hemorrhage, sometimes with inflammatory changes, were seen in the spleen; occasionally hemorrhages were seen in lymph glands; and in one instance, a striking hemorrhage was observed in the germinal centers of the lymphoid tissue of the small intestine. A spontaneous lymphoid tumor of the spleen, about 2.5 cm. in diameter, was found at necropsy. Portions of the tumor were hemorrhagic, perhaps an effect of the polysaccharide. Congestion of the liver was a prominent feature, and in a few instances hemorrhage was noted. Sometimes there was focal necrosis of liver cells. The changes in the kidney were usually slight, but congestion, hemorrhage, and the presence of casts in the tubules were observed. Severe hemor-

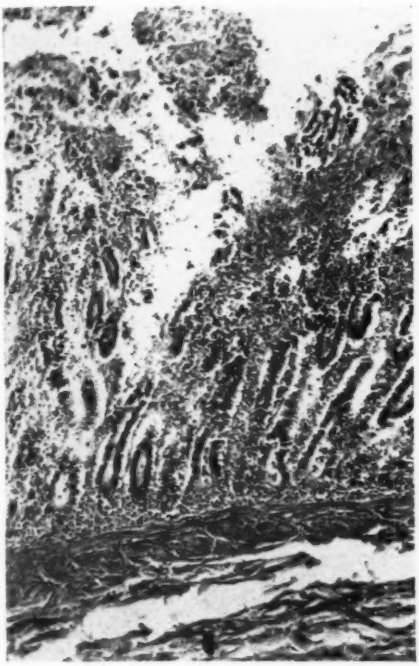

FIGURE 6.—Congestion, hemorrhage, and necrosis of mucous membrane of small intestine of dogs following injection of polysaccharide. × 50.

rhage in the adrenal medulla was found once, and subendocardial hemorrhage occurred in the five dogs necropsied after adrenalin therapy. The intestinal congestion and hemorrhage were consistently intense. The myocardium, lungs, and other organs examined showed little change. The pial vessels of the brain were congested in two dogs dying of convulsions, but gross hemorrhages were not found.

INJECTION OF POLYSACCHARIDE INTO GUINEA PIGS

The intracardiac injection of 0.1 to 0.3 mg. of polysaccharide per kilogram of body weight caused prostration in 1 and dyspnea in 3 of 22 guinea pigs. The majority showed no disturbance. A dose of 1.0 to 5.0 mg. per kilogram was fatal to 8 of 15 animals injected; death took place in 6 to 38 hours, the average time to death being 19 hours. Deeper respiration was the most frequent and most pronounced disturbance. It tended to disappear after the eighth hour or to persist until death. Prostration was rather characteristic: first the pelvis could not be supported, then the chest and abdomen rested on the floor, and lastly the head.

Usually no effects were observed following the subcutaneous injection of 0.1 mg. of the polysaccharide per kilogram body weight. With doses of 1.0 to 5.0 mg., respiratory stimulation and prostration were prominent. The nine guinea pigs receiving 10 mg. per kilogram died in 16 to 20 hours. Dyspnea and prostration with a loss of righting reflexes were typical. The rectal temperature sometimes fell. Convulsions were seen in each of the five guinea pigs

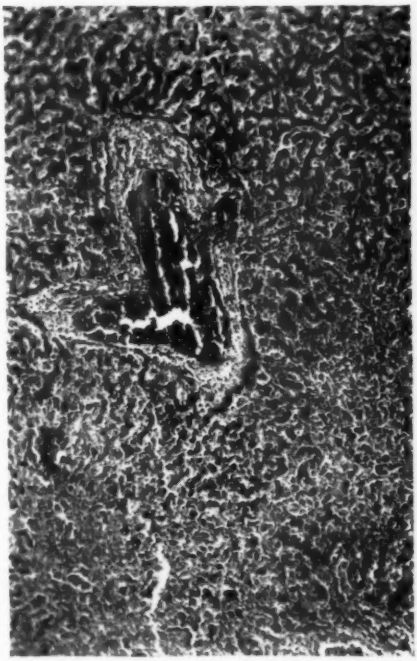

FIGURE 7.—Congestion and hemorrhage in liver of dog following injection of polysaccharide. × 60.

whose death was observed. These animals were necropsied soon after death. A wide area of congestion and edema of the subcutaneous tissues of the anterior abdominal wall at the site of injection were observed in all five. The congestion in some extended to the underlying peritoneum. Congestion of the liver, lungs, brain, and stomach were occasionally seen. No gross hemorrhages were observed in the brain.

EXPERIMENTS WITH RATS

The chief difference observed in the distribution of the dye in rats was that in three rats the site of subcutaneous injection of the polysaccharide was stained more deeply than was the site of the saline injection in the control. In one pair of rats, the reverse was true.

DISCUSSION

The L. D. 50 of the polysaccharide administered to the guinea pig subcutaneously is about 5 mg. per kilogram body weight. The experiments on the unanesthetized dog are too few to establish the L. D. 50 but do indicate a similar order of toxicity. A higher percentage of deaths was noted when comparable doses of the polysaccharide were injected intraperitoneally than when given subcutaneously.

When a lethal dose of the polysaccharide was injected into unanesthetized dogs and guinea pigs, the animals usually died between 12 and 24 hours after injection. The anesthetized dogs would die in 1 to 4 hours. After a lethal dose of the polysaccharide, the unanesthetized dog typically showed retching, vomiting, diarrhea, a rise in body temperature, and finally convulsions. These symptoms were not displayed by the anesthetized dog. Because anesthesia so greatly modifies the effects of the injection, experiments were carried out which would not require the use of an anesthetic even though desirable experimental procedures would have to be omitted.

In the experiments carried out under anesthesia, circulatory failure was evidently the cause of death. The respirations seemed adequate until the blood pressure reached a very low level, thus reducing the blood supply to the respiratory center. If the dog did not die in a few hours, the blood pressure gradually rose, approaching the normal level. The effects of the anesthetic began to wear off about this time, and the dog would recover consciousness. Two unanesthetized dogs showed only a small drop in blood pressure at the time when 12 of 14 anesthetized dogs showed a great drop in blood pressure. This fact indicated that the anesthetic played an important role in the fall in blood pressure, perhaps by interfering with compensatory vasomotor responses. In view of the other evidence, the decrease in pulse pressure probably indicates a decrease in cardiac output.

Adrenalin therapy was unsuccessful in preventing the fall in blood or restoring it to normal. Continuous intravenous injection might have been more helpful. The amount used was large (*4, p. 434*), and it is not unlikely that it would be an advantage to give more adrenalin. Adrenalin alone can produce a condition resembling surgical shock (*5, 6*); it can also produce congestion, hemorrhage, and ulceration of the small intestine (*7*), and subendocardial hemorrhages. Adrenalin administered after polysaccharide injection augmented the intestinal congestion and hemorrhage and the subendocardial hemorrhages. Possibly an excessive secretion of adrenalin, perhaps combined with sympathetic vasoconstrictor impulses, is the mechanism whereby the polysaccharide produces the lesions mentioned.

The similarity of the clinical picture to parathyroid tetany is striking, but the serum calcium showed no significant change following administration of the polysaccharide. It is conceivable that the respiratory stimulation so frequently seen might produce an alkalosis by blowing off carbon dioxide, thus decreasing the ionization of blood calcium and giving rise to tetany. We have no direct evidence on this point.

Apparently the stimulation of the central nervous system was widespread. Clonic convulsions were more frequently tonic in type, indicating involvement of subcortical centers. The rigid extension of the legs and the opisthotonos remind one of decerebrate rigidity. Unlike decerebrate rigidity, the condition soon passed off. The extensor reflexes were active or hyperactive; reflex figures could be elicited. The twitching of the ears and facial muscles, the clenching of the jaws, the salivation, and the constricted pupils indicated that supraspinal mechanisms were stimulated.

The rise in body temperature, in nonprotein nitrogen of the blood, and the respiratory stimulation were possibly brought about by increased catabolism. The escape of red blood cells into the tissue spaces, apparently by diapedesis, implied abnormal permeability of the minute blood vessels. The tests of capillary permeability in rats and the tests of capillary fragility in the dog did not show striking differences from the normal. Transudation of fluid into serous cavities was seen only once at necropsy. The excessive thirst and fall in blood pressure could be explained by decreased blood volume. The low blood pressure and decreased blood volume seen in surgical shock are associated with diminished reflexes, but these animals injected with polysaccharide showed exaggerated extensor reflexes and convulsions.

SUMMARY

Injection of the polysaccharide obtained from *Serratia marcescens* (*Bacillus prodigiosus*) into anesthetized dogs resulted in death from circulatory failure in a few hours when doses from 0.3 to 4.3 mg. of the material per kilogram of body weight were given intravenously.

Adrenalin was not effective in maintaining or restoring the blood pressure.

Injection of a lethal dose of the polysaccharide into unanesthetized dogs resulted in convulsions and death usually between 12 and 24 hours later.

Congestion of the liver, congestion and hemorrhage in the intestinal mucosa and in the lymphoid tissue of the small intestine, and lymph glands were found at necropsy, following administration of lethal or sublethal doses.

An increase in capillary permeability was observed at the site of injection of the polysaccharide in the rat, with negligible effects elsewhere.

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CHEMICAL TREATMENT OF TUMORS. X. REACTIONS OF FOUR PATIENTS WITH ADVANCED MALIGNANT TUMORS TO INJECTION OF A POLYSACCHARIDE FROM *SERRATIA MARCESCENS* CULTURE FILTRATE[1,2,3]

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It is by now well established that certain preparations of bacterial origin, when administered parenterally to animals bearing sarcomas, produce hemorrhage in the tumors within a few hours. The action is highly selective, for while severe hemorrhage is induced in the tumor, it has not been reported to occur in any of the normal tissues (*2–5*), with a few minor exceptions (*6*) of a slight degree. The hemorrhage and necrosis produced in this way are followed, in some instances, by complete and permanent regression of the tumor.

The bibliography[5] on the treatment of human tumors with induced bacterial infections or with bacterial products began about 75 years ago (*11*). Fehleisen's paper (*12*) on the effect of cultures of the streptococcus of erysipelas upon human tumors is better known. In 1888 Bruns (*13*) published a critical review of the literature on clinical experiences, with 27 references. Coley's first paper (*14*) appeared in 1891; and from that time until his death in 1936, he published many papers dealing with the results obtained on treating patients with a preparation made from cultures of *Erysipelas streptococcus* and *Bacillus prodigiosus*, commonly referred to as "Coley's mixed toxins." His last publication (*15*) contains a summary of his work with this preparation.

In 1910 Loeb (*16*) reviewed the evidence as to its value, and the Council on Pharmacy and Chemistry of the American Medical Association (*17*) included "Erysipelas and Prodigiosus Toxins (Coley)" in its list of New and Nonofficial Remedies. In 1934 the merits of this preparation were surveyed again, and the council (*17*) retained it on its list—

> with a view to facilitating further studies with the product, especially in connection with its use as a prophylactic in conjunction with conservative or radical surgery. Its use in definitely inoperable cases may be quite justified, in many instances, as a desperate attempt to combat the inevitable.

This preparation was, however, dropped from the list in 1942.

For a time clinicians occasionally employed this preparation, but it never gained wide use because in most cases no effect on the neoplastic process was noted. Reports of complete regression of tumors following treatment with this agent were rare and constituted only a very small percentage of the cases treated. Moreover, its administration was frequently attended by high temperature, nausea, and vomiting.

Interest in the effect of bacterial preparations on tumors in experimental animals was revived in 1931 by the report of Gratia and Linz (*18*). Their work was soon confirmed and was extended in several directions by a number of other investigators. (See footnote 5.) Because of the high toxicity of such bacterial preparations, Shear and Andervont (*19*) separated the active agent from toxic and inactive constituents; from filtrates of broth cultures of *Bacillus coli* a polysaccharide fraction was obtained (*20*) which produced hemorrhage in transplanted sar-

[1] This work was carried out in Boston in 1939 while one of us (A. M. B.) was associate physician, Medical Clinic of the Collis P. Huntington Memorial Hospital, Boston, and the other (M. J. S.) was stationed at the Office of Cancer Investigations, U. S. Public Health Service, then located at the Wolcott Gibbs Memorial Laboratory, Harvard University, Cambridge.

[2] Presented at the 1940 meeting of the American Society for Clinical Investigation (*1*).

[3] This is paper No. 599 of the Cancer Commission of Harvard University.

[4] The authors wish to thank Drs. Joseph C. Aub, Floyd C. Turner, Rulon W. Rawson, and Shields Warren for their cooperation in this study. The chemical determinations of blood and urinary constituents were made by Drs. Jytte Muus and Hildegarde Wilson.

[5] The literature has been summarized in earlier papers of this series (*7–9*) and in ch. VIII of Shwartzman's monograph on local tissue reactivity (*10*).

comas in mice with doses of 0.4 microgram of the active material. Shear and Turner (*8*) obtained a similar polysaccharide fraction from filtrates of cultures of *Serratia marcescens* (*B. prodigiosus*), one of the organisms used in the preparation of "Coley's mixed toxins."

Comparative assays (*8*) in mice bearing transplanted sarcomas gave the following results, expressed in number of mouse tumor units[6] per cubic centimeter: A commercial specimen of Coley's mixed toxins, 100 units; untreated filtrates of cultures of *Serratia marcescens*, 330 units; and a 1-percent solution of the polysaccharide from filtrates of *S. marcescens*, 100,000 units.

Four patients with diverse types of malignant tumors were selected for an exploratory study of their reaction to the administration of the polysaccharide fraction prepared by Shear and Turner (*8*). All these patients had inoperable tumors and widespread metastatic growths which were no longer sufficiently susceptible to radiation to justify further use of radiotherapeutic measures.

PROCEDURE

During the period of study, the patients were maintained on a light hospital diet. For 5 days, following each injection of the polysaccharide, the food intake was restricted to between 40 and 50 gm. protein, chiefly in the form of milk; the ration thus contained minimal amounts of purine nitrogen. Twenty-four-hour collections of urine were made in all cases during this 5-day period. The urine was analyzed for total nitrogen by the micro-Kjeldahl method, and for uric acid by Folin's molybdate method after preliminary precipitation as silver urate. At frequent intervals complete blood counts were made, and the blood was analyzed for nonprotein nitrogen and for uric acid. In one case, determinations were also made of plasma protein.

A sterile solution of the polysaccharide fraction (*8*), containing 40,000 m. t. u. per cubic centimeter, was tested for toxicity in three species of animals (mouse, guinea pig, and monkey). The animals survived doses, per kilogram of body weight, considerably in excess of those subsequently given to the patients.

This solution was injected intramuscularly except in one instance, when it was given intravenously, in doses ranging from 0.15 to 0.6 cc., into the four patients selected. Necropsies were obtained in the three cases in which death occurred in the hospital about 1 month after the initial injection. The fourth patient was discharged after 3 weeks. When he died 6 months after the single injection, a necropsy was not obtained.

CASE REPORTS

Case 1 (*H. H. 39–888*).—Adenocarcinoma of the prostate with widespread metastases to bone.

The patient was a 71-year-old leather worker (white male). Two years previously, a diagnosis of prostatic carcinoma with metastases had been made. Six months later, he was given 1,200 r units of 200-kv. X-radiation to each of three sites in the lumbar spine and pelvis, and a suprapubic cystotomy was performed. This was followed a month later by a transurethral prostatic resection to re-establish urinary flow. Four months later, he received 1,200 r units of X-ray to each of two sites. After another 6 months, he was given further radiation over two sites as before, and a second suprapubic cystotomy was performed. Six weeks prior to treatment with bacterial polysaccharide, roentgenograms at Pondville Cancer Hospital, Wrentham, Mass., showed extensive metastatic involvement of the pelvis, spine, ribs, and femurs; further X-ray therapy was considered unwise. He was kept relatively free from pain with 15 mg. morphine (or its equivalent) two to three times daily.

On admission to Huntington Hospital, physical examination showed an emaciated man, unable to stand or walk, in great pain. There was complete paralysis of the external rectus muscle of the left eye. A hard lymph node was felt in the left supraclavicular area. The heart was slightly enlarged to the left, the blood pressure was 144/88, and the pulse rate was 94. The lungs appeared normal. Urine was draining through a supra-

[6] A mouse tumor unit (m. t. u.) (*21*) was defined (*8*) as the amount which, under standard conditions, regularly produced hemorrhage in 50 percent of the mouse tumors.

pubic cystotomy tube and, on rectal examination, a hard, enlarged mass was felt in the prostatic area.

On the next afternoon, 0.2 cc. (8,000 m. t. u.) of the polysaccharide was given intramuscularly. One and one-half hours later, the patient had a mild chill, and his temperature rose slowly. Four hours after the injection he complained of extremely severe pain in the right hip, which he described as worse than any other pain that he had ever experienced. He had a severe chill at this time, his pulse rose suddenly to 150, and his blood pressure fell to 70/50. The rectal temperature reached a peak of 103° 6 hours after the injection and then decreased slowly. Accompanying this drop in temperature, the pulse rate diminished to 100, the patient became irrational, basal râles were heard, and pitting edema of the ankles and over the sacrum appeared. The systolic blood pressure remained below 100 mm. of mercury. Eighteen hours after the injection, he became anuric. Twelve hours after this, following intravenous administration of hypertonic glucose and intramuscular neosynephrin, the blood pressure rose to 110/70, and urine began to flow slowly. During most of the next week he was unconscious or irrational and had generalized edema and congestive heart failure which slowly improved. Two lumbar punctures showed only moderately increased pressure (170 mm. water).

For the 48 hours after treatment, dilaudid gr. 1/32 was given nine times. During the next 3 days no sedation was required inasmuch as the patient was unconscious; it was then required about twice daily for 1 week, and once daily for another week. During the last 2 weeks of his stay at the hospital, it was given only twice, and then to ease general discomfort.

During the second and succeeding weeks after admission, he slowly gained in strength and appetite; he sat up on the eighteenth day and began to walk around freely on the twenty-fourth day. No further injection of the bacterial preparation was given. He was discharged to a nursing home on the twenty-eighth day. During the next 2 months, he remained ambulatory and free from pain and was seen twice in the out-patient clinic. Pain then recurred; he became bedridden and died 6 months after the concentrate was given. No necropsy was obtained.

The supraclavicular node appeared to enlarge during the reaction which followed the injection of the polysaccharide, and a biopsy was done on the tenth day. Dr. Shields Warren made a diagnosis on the specimen of metastatic adenocarcinoma, without evidence of hemorrhage.

X-ray examination of the lumbar spine and pelvis showed widespread osteoplastic and destructive metastases, which did not change in appearance during the 2 months following treatment. The laboratory findings are detailed in figure 1.

Following a single injection of the polysaccharide from *Serratia marcescens* culture filtrate, this patient with advanced prostatic carcinoma had a moderate febrile response followed by a prolonged fall in blood pressure, a brief period of anuria, and signs of congestive heart failure lasting about 10 days. Blood nonprotein nitrogen and uric acid rose to a high level and fell slowly. There was acute pain in areas of bone metastasis for a few days after the treatment, after which there was complete relief from pain which lasted for over 2 months. The emaciated, bedridden patient gained strength and became ambulatory again for several months. Death occurred 6 months after the injection.

Case 2 (*H. H. 39-964*).—Multiple myeloma.

The patient was a 47-year-old housewife (white). A roentgenologic diagnosis of multiple myeloma involving most of the bones, probably arising in the second lumbar vertebra, had been made 19 months previously. She had been bedridden 6 months because of collapse of the second lumbar vertebra and a pathologic fracture of the right humerus.

At the time of admission, physical examination showed palpable hard tumors over the skull, manubrium, and three ribs, marked kyphosis in the upper lumbar region, and trophic ulcers over the ilia. The heart and lungs appeared normal on examination; the blood pressure was 130/68.

On the third day after admission, 0.15 cc. (6,000 m. t. u.) of the polysaccharide solution was given in the right deltoid region. Four hours later there was a moderately severe chill, and the temperature rose abruptly to 103°; it returned to normal 5 hours later. Blood pressure at the height of the fever was 84/60 and remained below 100 systolic for several hours. During the next 4 days, it slowly regained the admission level.

At the height of the febrile reaction, fine, moist râles were heard at the bases of the lungs. The next day these had given way to coarse râles, although no other signs of cardiac decompensation appeared. A few type 20 pneumococci were cultured from the sputum. For 4 days she was mentally depressed and agitated, with irrational periods. Because of anemia, a blood tranfusion was given 11 days after treatment with the polysaccharide. A week later, low-grade fever appeared, and an intense apical presystolic murmur was heard. The fever increased slowly, pathologic fractures occurred in the sternum and ribs, and she died 4 weeks after admission.

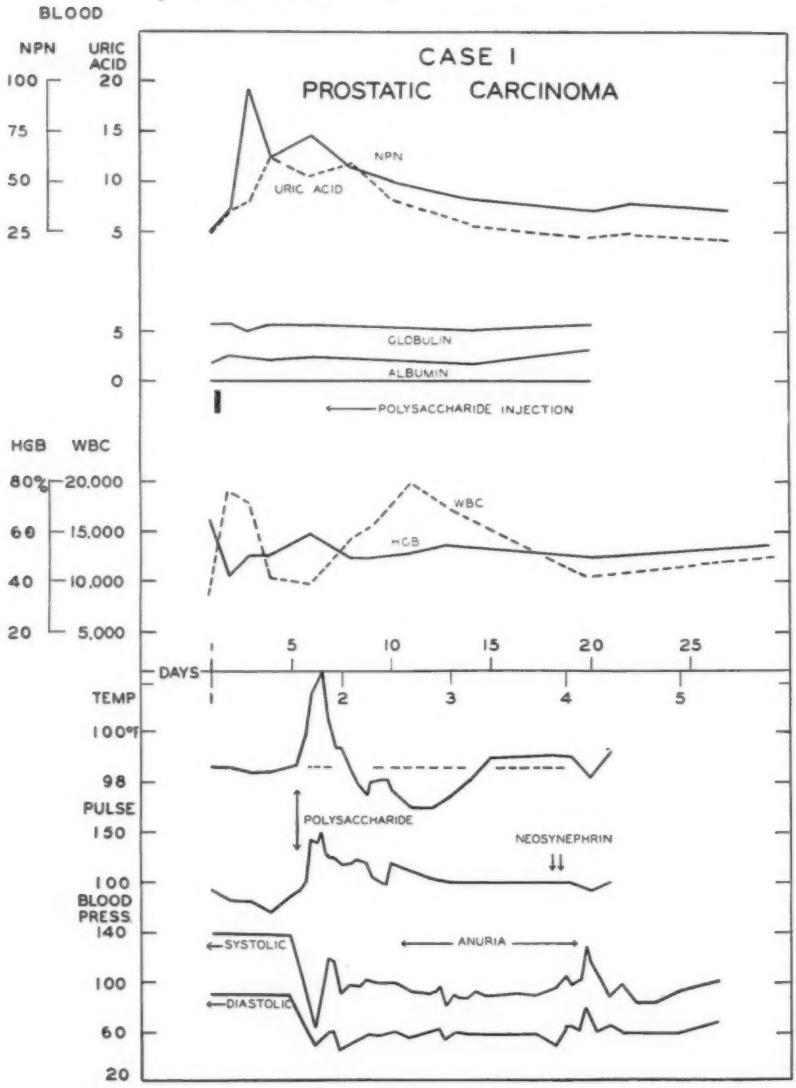

FIGURE 1.—Effects in case 1 of one intramuscular injection of a polysaccharide from *Serratia marcescens*, expressed as follows:

UPPER PART

The time scale represents a period of 30 days;
Blood nonprotein nitrogen and uric acid in milligrams per 100 cc. of blood;
Albumin and globulin in grams per 100 cc. of plasma.

LOWER PART

The time scale represents a period of 5 days;
The pulse rate is the rate per minute;
Systolic and diastolic blood pressures in millimeters of mercury.

Necropsy by Dr. Charles E. Dunlap showed multiple myeloma, involving ribs, spine, pelvis, sternum, skull, lungs, and adrenals. Diffuse hemorrhage was seen in a few of these tumors. There were arteriolar nephrosclerosis and moderate coronary sclerosis, and an acute vegetation was found on the mitral valve, from which no organisms were obtained. The laboratory findings appear in figure 2.

Administration of a polysaccharide fraction from a culture filtrate of *Serratia marcescens* to this patient with multiple myeloma produced a moderate febrile reaction, transitory hypotension, and dependent pulmonary congestion. Death 4 weeks later followed the development of endocarditis and pathologic fractures of the ribs and sternum. There was no evidence that the course of the disease was altered by this treatment.

Case 3 (*H. H. 39–228*).—Lymphosarcoma.

The patient was a 26-year-old white male textile worker, who first noted enlarged cervical nodes 2 years previously. These nodes regressed following X-ray treatment and then recurred. A biopsy showed lymphosarcoma, and further X-ray treatment was given. Ten months later, metastatic osseous lesions in the right leg were demonstrated but disappeared following local radiotheraphy. Ten months after that, painless jaundice occurred and was treated with 500 r units of 1,000-kv. X-ray, given anteriorly and posteriorly to the abdomen. During the following 4 months, generalized lymph-node enlargement and splenomegaly appeared, and lesions occurred in the kidneys, central nervous system, skull, and pelvis. These lesions were all temporarily improved by a total of three courses of X-radiation. On his final admission, the patient complained of severe pain in the pelvis, tinnitus in the left ear, and pain and weakness in the left arm. There were many enlarged nodes in the neck and both axillae. X-radiation (600 r units) was given to the left axilla, but pain in the arm grew worse, cerebral signs recurred, and Horner's syndrome of the right eye was noted. He gradually became stuporous, and 8 days after the final radiation treatment he lapsed into a coma. The temperature rose steadily to 103°.

Ten days after completion of X-ray therapy, he was considered to be dying. At this time, 0.2 cc. (8,000 m. t. u.) of the polysaccharide solution was given intramuscularly into the right deltoid. No chill was noted, but in 4 hours the temperature had risen to 107.4°, and the systolic blood pressure had fallen from 110 to 80 mm. Heart sounds were normal, and there were no signs of decompensation. The temperature fell gradually, but there were occasional rises between 105° and 107°. A transfusion was given the day after administration of the polysaccharide. On the second day he responded slightly; by the seventh day the temperature did not go above 100°, and he was rational.

All lymph-node enlargements had disappeared, except that in the right side of the neck, and the enlargement there had decreased in size. Pain in the arm was less severe than before. Two weeks after treatment, intracutaneous inoculation was given; 0.05 cc. of a 1 : 100 dilution of the polysaccharide solution (20 m. t. u.) was used in order to determine sensitivity before giving another injection. There was no immediate reaction, but in the course of 2 days a large indurated area about 4 cm. in diameter appeared at the site, resembling a positive tuberculin reaction. A regional lymphangitis appeared at the same time. This reaction subsided after the second day. At this time, a second injection, 0.2 cc. (8,000 m. t. u.), of the polysaccharide solution was given into the left deltoid; the temperature rose gradually to 103° after 6 hours. The pulse rose correspondingly, but there was no change in blood pressure. The temperature remained above 101° for 16 hours and then returned to normal. On the following day, at the site of injection there was extreme tenderness which continued to be noticeable for 3 days.

Following this, there was a gradual increase in the earlier symptom of pain in the left arm, and in addition pain appeared in the right groin. The patient was then given a third injection (of 0.3 cc.) of the polysaccharide into the right quadriceps femoris. The reaction was even more gradual; the temperature reached 103° after 14 hours and then fell slowly. A fourth injection, 0.4 cc. (16,000 m. t. u.), was given into the opposite quadriceps 5 days later. This injection was followed by a mild general response. There was great local tenderness after both of these injections. In the meantime, symptoms began to recur; the weakness of the arms increased, and the lmyphadenopathy slowly reappeared. It was therefore thought advisable to try the effect of intravenous injection, and on the thirty-first day after the original injection, 0.1 cc. (4,000 m. t. u.) of polysaccharide, diluted to 30 cc. with saline, was given intravenously (about 5 cc. per minute.) Ten minutes later the patient complained of severe precordial pain radiating down the left arm and also of abdominal pain. In the course of a few minutes he became hyperpneic and cyanotic. The pulse rose to 170, and the temperature (axillary) to 106°. Death because of respiratory failure occurred about 1½ hours after the injection.

Post-mortem examination by Dr. Olive Gates showed lymphosarcoma involving many

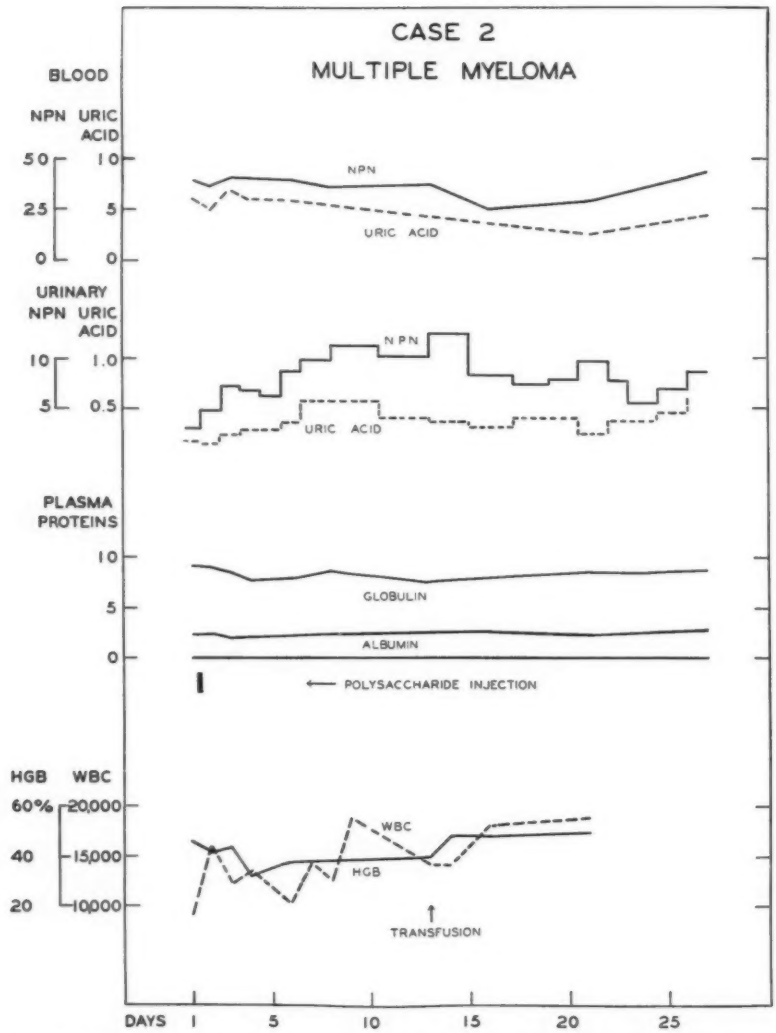

FIGURE 2.—Response of case 2 to one intramuscular injection of a polysaccharide from *Serratia marcescens*. The urinary nonprotein nitrogen and uric acid are given in terms of grams per 24-hour period. The other quantities are expressed in the same units as in figure 1.

lymph nodes and the cerebellum, skull, adrenals, kidneys, pituitary, and thyroid. The coronary vessels were normal. The lymph node in the right axilla contained a central focus of hemorrhage; no gross blood was seen in the other tumor areas. There was slight pulmonary congestion and edema. For the laboratory findings, see figure 3.

This patient, who had widespread lymphosarcoma and considerable intracranial involvement, was treated with the polysaccharide when apparently in terminal coma. After a long period of hyperpyrexia, he recovered consciousness, and several enlarged peripheral lymph nodes were no longer palpable. Subsequent to the first injection, there was a distinct rise in blood nonprotein nitrogen and uric acid, and several grams of uric acid were excreted in the next 4 days. A skin test with a diluted solution 2 weeks later gave a tuberculinlike reaction associated with lymphangitis. Three succeeding injections of the concentrate in increasing doses gave mild reactions, while tumors gradually recurred. Following an intravenous injection of the concentrate, precordial pain was noted, the temperature and pulse rose extremely rapidly, and death occurred because of respiratory failure. Necropsy showed a fresh hemorrhage in the center of one enlarged lymph node.

Case 4 (*H. H. 39-197.*)—Ewing's tumor.

The patient was a 25-year-old physician. He had noted pain in the left hip 10 months before admission for treatment with the polysaccharide; 3 months later, biopsy established the diagnosis of Ewing's tumor involving the left ilium and ischium. Five thousand r units of 1,000-kv. X-ray were administered over this lesion, three fields being used. After this treatment, X-ray films showed some recalcification of the bony defect. Two months later a metastasis appeared in the left humerus, which regressed somewhat following X-ray treatment. During the next 3 months lesions appeared in the skull, spine, scapula, ribs, and lungs, all of which were treated with large doses of 1,000-kv. X-ray locally.

At the time he was admitted to the hospital, there were sharply localized areas of tenderness in the sixth rib on the right side and in the left ilium. A physical examination gave negative findings otherwise. Injection of 0.2 cc. (8,000 m. t. u.) of the polysaccharide solution intramuscularly evoked a chill and a rise in temperature to 103° which fell slowly for 24 hours. There was no marked change in blood pressure. The areas of tenderness noted on his admission to the hospital became extremely painful and then after 2 days subsided altogether. Two weeks later an intracutaneous skin test with a 1:100 dilution of the concentrated solution was given. The response, as in the previous patient, consisted in the slow development of local induration and lymphangitis, with enlargement of an adjacent node.

A second injection of 0.2 cc of the polysaccharide was then given. During the succeeding 18 days, 3 further injections were given in increasing dosage up to 0.6 cc. (24,000 m. t. u.), all of which gave reactions similar to the first. While these treatments were being given, headaches and tenderness over the cranium occurred, and a total of 225 r units of 1,000-kv. X-ray was given over the skull. The patient complained of pleuritic pain in the region of the seventh rib on the left side, which was treated similarly with 350 r units of X-ray. A few days before the last injection, a bloody postnasal discharge was found to be coming from the ethmoid region. Two days after the last injection, the patient showed considerable dyspnea and signs of pulmonary consolidation. This condition grew steadily worse, and death occurred 3 days later. During the last few days small diffuse ecchymoses were observed at sites of hypodermic injections. Post-mortem examination by Dr. Shields Warren and Dr. H. E. MacMahon showed Ewing's tumor, involving the left ilium, temporal bone, sphenoidal and posterior ethmoid sinuses, dura, lungs, lymph nodes, and vertebrae. Many of the tumors were hemorrhagic. For the laboratory findings, see figure 4.

A patient with Ewing's tumor of the ilium with widespread metastases had two painful lesions at the time of the first injection of the polysaccharide. The first injection produced severe local pain in these two areas, followed by complete relief from pain. No subsequent response, aside from initial chill and fever, was elicited by four additional injections in increasing dosage. New metastatic lesions appeared rapidly during the course of treatment.[7]

DISCUSSION

The administration of a polysaccharide obtained from culture filtrates of *Serratia marcescens* produced several effects.

[7] Other aspects of this case have been discussed by Roberts (22).

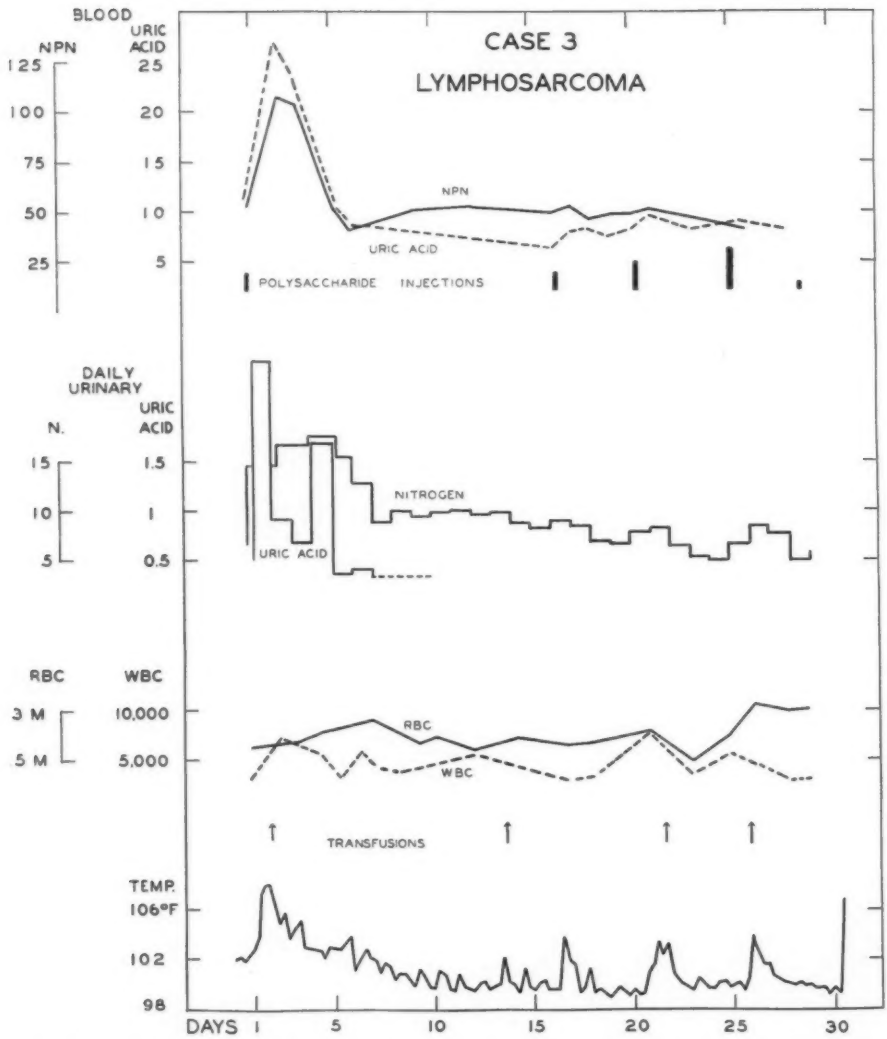

FIGURE 3.—Response of case 3 to four intramuscular injections of a polysaccharide from *Serratia marcescens*. The final dose was given intravenously.

FEBRILE REACTION

Injection of the polysaccharide was usually followed by a marked chill 1 to 4 hours later. The temperature rose steadily after injection to a peak about 4° F. above the previous level, in 4 to 10 hours, and then fell to normal within 6 to 24 hours. During the febrile period, pain in the back and extremities was noted.

BLOOD PICTURE

Leukocytosis occurred regularly for about a day following injection, reaching approximately double the level on admission. This was generally associated with the appearance of 1 to 5 percent of young cells of the myeloid series. In the patient with lymphosarcoma, however, a sharp increase in the relative number of lymphocytes was noted. The red cell count and hemoglobin values fell 15 to 25 percent during the first 2 days after the first injection. The interpretation of this finding is not clear; since serum protein values showed no parallel change, it was probably not ascribable to dilution of the blood following hemorrhage. No evidence of blood destruction in the form of increased plasma or urinary pigments was observed.

Sedimentation rates were extremely high subsequent to treatment (up to 8 mm. per minute in two patients). Although this represents an increase during treatment, the data are insufficient to show the magnitude of the increase.

The two patients who received repeated doses eventually showed a marked decrease in blood platelets and a tendency to bleed. Both of these patients, however, showed involvement of bone marrow by tumor at the time of necropsy, so this finding was not considered necessarily related to the treatment. However, Shimkin and Zon (*23*) later found that this polysaccharide, injected into mice bearing subcutaneous implants of sarcoma 37, produced a sharp drop in the platelet count in 6 hours; no significant change in the red-blood-cell count was observed in this period.

CARDIOVASCULAR EFFECTS

Three of the four patients showed a marked fall in blood pressure, beginning usually at the height of the febrile reaction. In the first case, it seemed probable that the hypotension contributed to the development of anuria. The two older patients developed signs of cardiac failure (pulmonary congestion and peripheral edema) beginning during the hypotensive period. The younger patients, who undoubtedly had a greater cardiac reserve, did not show any evidence of congestive failure. On injecting into normal dogs specimens (*8*, *24*) of this polysaccharide that had been purified further, Franke and Richert (*25*) obtained electrocardiograms that were essentially negative except for "cardiac acceleration, a sinus tachycardia." Even when sublethal doses were given, according to these workers:

> There were no ectopic beats and no disturbance in atrioventricular conduction. * * * No significant changes, except the development of a sinus tachycardia, were observed in the electrocardiograms of dogs that had received sublethal doses * * *.

SENSITIVITY TO THE MATERIAL

The first injection produced moderate local tenderness. Subsequent injections gave rise to considerably greater local pain and tenderness. Intracutaneous inoculation of a 1:100 dilution of the concentrated solution, following intramuscular administration, gave a marked inflammatory reaction characterized by a wide area of induration, and in one case local lymph-node enlargement. This patient (case 3) died within 2 hours after a much-reduced dose was given intravenously. Although the extent of the intracranial involvement was adequate to explain the respiratory failure, this fact probably does not account for the severe precordial pain noted.

Shwartzman (*26*), working with crude filtrates of *Serratia marcescens* and a trypsin-treated specimen (*8*, *24*) of the polysaccharide prepared by

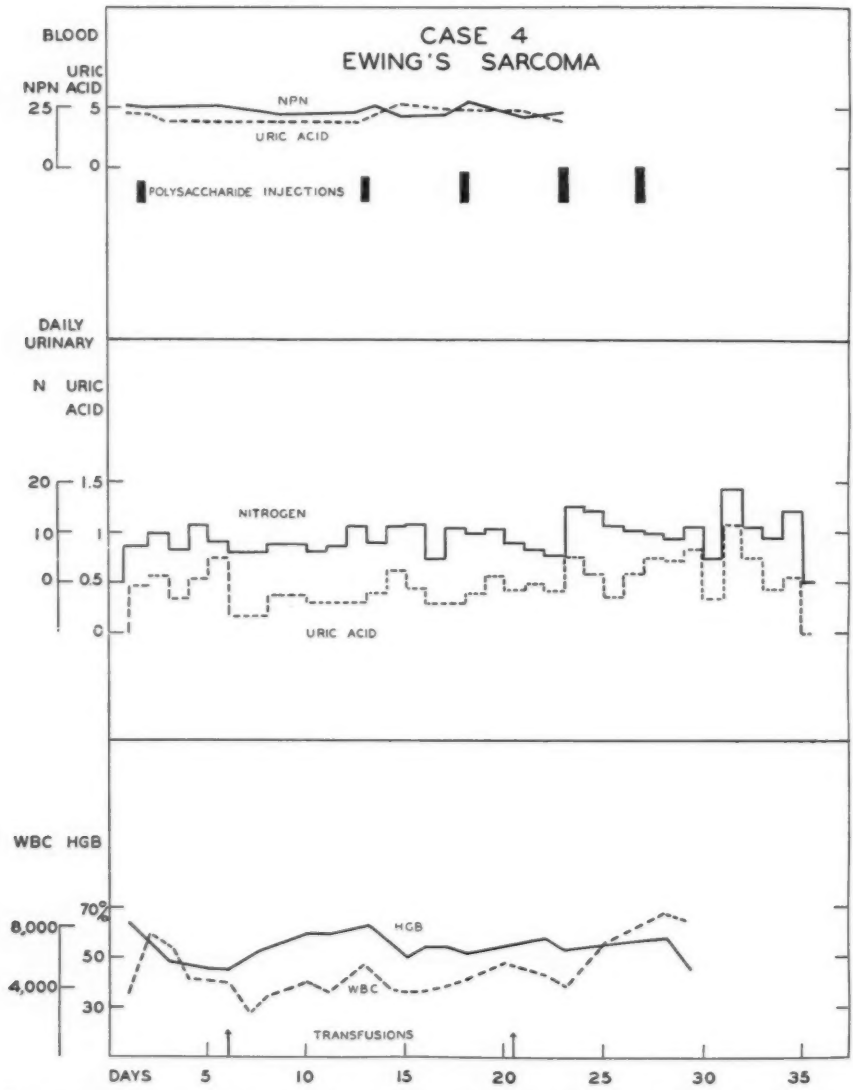

FIGURE 4.—Response of case 4 to five intramuscular injections of a polysaccharide from *Serratia marcescens*.

Shear and co-workers, found that both were capable of eliciting the phenomenon of local skin reactivity in normal rabbits. The filtrates and the polysaccharide were both found to be highly antigenic; following a course of immunizing reactions in rabbits, they both stimulated production of antiserums that gave precipitin reactions with the filtrates and polysaccharide and neutralized the active principles. Cross-precipitation and cross-neutralization experiments showed a close antigenic relationship to exist between both materials. Franke (*27*) attempted to produce anaphylaxis in guinea pigs with the polysaccharide (*8*, *24*) without success. He stated:

> While some doubtful reactions were obtained, anaphylaxis was not clearly demonstrated in any of the 64 guinea pigs injected with a sensitizing and with a shocking dose.

With a large enough dose, Franke (*28*) found that death in the dog resulted from circulatory failure. A dose of 1 mg. per kilogram was required to kill the unanesthetized dog; in the dose range 1 to 8 mg. per kilogram, 6 of 21 dogs recovered. With an intravenous dose of 1 to 2 mg. per kilogram, 2 of 3 dogs recovered. In many of the dogs that died the heart continued to beat for a short period after respiration had ceased.

Since this polysaccharide contains about 10,000 m. t. u. per milligram, the intravenous injection of 4,000 m. t. u., which was given to 1 patient, corresponded to 0.4 mg. of active polysaccharide. For a patient weighing about 50 kg., this dose would be about 0.01 mg. per kilogram, which is far below 1 mg. per kilogram, the L. D. 50 for unanesthetized dogs and guinea pigs (*27*, *28*).

EVIDENCE OF TISSUE BREAKDOWN

The marked and rapid increase in uric acid and nonprotein nitrogen in the blood of two patients and the increased excretion of these substances, as demonstrated in one patient, suggested that an acute tissue breakdown had taken place. It is of interest that the relief of some symptoms ran parallel to this chemical evidence and to the severity and duration of the systemic response to the polysaccharide. Whether this inferred tissue breakdown by the agent was restricted to the tumors is another of the open questions.

A relatively high proportion of the excess nonprotein nitrogen of the blood in these patients was in the form of uric acid. Thus in case 1 (disregarding the anuric period), the nonprotein nitrogen of the blood exceeded the basal level by 55 mg. percent while the excess of uric acid nitrogen was 2.4 mg., i. e., 4.4 percent of the total excess nitrogen. In case 3 the corresponding figures were: 65 mg. excess nonprotein nitrogen; 5.0 mg. excess uric acid nitrogen, or 7.7 percent of the total excess nitrogen. In the same patient an excess of 33.6 gm. of urinary nitrogen over the resting level appeared during the 4 days following treatment. Of the total excess nitrogen, 1.4 gm. was uric acid nitrogen: this gave a value of 4.2 percent for the ratio of uric acid nitrogen to total nitrogen. The smaller increases which occurred in case 4, and after repetition of injections in case 3, were not great enough to justify calculation of the ratio. The foregoing ratios raise the question whether a large proportion of the protein presumed to have been broken down was nucleoprotein. Such a process would be similar to that sometimes observed in the therapy of leukemia by radiation (*29*) and to the much slower process seen following radiation therapy of other tumors (*30*, *31*).

The two patients showing rapid increases in blood nitrogen had urinary-tract complications. In case 1 the period of anuria, which was related to the hypotension, may have been responsible for these increases. In patient 3, however, large quantities of uric acid appeared in the urine in spite of renal involvement by tumor.

In any event, the systemic response, including hypotension, of the first three

patients may well have been produced or magnified by the presence in the blood stream of breakdown products of tissue constituents. To what extent the reactions observed were a consequence merely of a nonspecific febrile reaction remains for future work to clarify. Although bacterial toxins alone may produce shock (*33*), previous observations (*9*) showed that much smaller doses of the polysaccharide are lethal in the presence of susceptible tumors than in their absence. A similar relationship holds in the case of colchicine, which also causes hemorrhage in tumors (*33*). Further physiologic studies will be necessary for an understanding of the shocklike state produced in tumor-bearing patients and animals (*9*). Any chemotherapeutic agent which causes sudden and massive destruction of tumor tissue, in contrast with the much slower tissue breakdown following radiation therapy, might be expected to have clinical effects on this basis. For the present it is considered possible that a major medical problem may be presented by any form of therapy causing rapid destruction of considerable amounts of tumor tissue.

ACTION ON TUMORS

In patient 3 several enlarged lymph nodes remote from the site of recent X-ray therapy were seen to regress, and it was found that one of the numerous remaining tumors had developed a hemorrhage. In patient 4 hemorrhages in tumors were seen, but regression of tumors was not obvious. Whether the hemorrhages were related to the therapy is still an open question. In the case of patient 1 evidence of symptomatic relief of pain was limited to areas containing metastases in bone. In patient 2 there was no clinical or post-mortem evidence that the polysaccharide had any effects aside from the induction of febrile reactions. In all cases, the lapse of weeks between first treatment and necropsy made it impossible to determine whether the first treatment produced hemorrhages similar to those seen in animal tumors a short time after injection.

The usual route of administration of Coley's mixed toxins has been the intramuscular; hence this was the method of injection employed in this preliminary study. However, since it was found (*9*) that subcutaneous injection of this agent had far less effect on mouse tumors than the intraperitoneal (or intravenous), a similar situation may be found to occur in patients. This remains for future work to determine.

The findings in this preliminary study do not warrant the conclusion that this polysaccharide should be recommended in the treatment of patients with malignant tumors. The reactions in two cases, taken in conjunction with the results of animal experimentation, suggest that further investigations may yield interesting results. However, great caution must be exercised even in the experimental employment of this material in patients. Small doses may provoke violent reactions, and death may follow in a short time unless precautions are taken to counteract the undesired responses. Further work on the latter point is now in progress.

SUMMARY

Four patients with advanced malignant tumors (prostatic carcinoma, lymphosarcoma, multiple myeloma, and Ewing's sarcoma) were given intramuscular injections of a polysaccharide from culture filtrates of *Serratia marcescens* (*Bacillus prodigiosus*) which produces hemorrhage in and partial or complete destruction of sarcomas in mice. All these patients died with tumors. Two patients showed noteworthy relief of symptoms. Two showed evidence of hemorrhage in tumors at post-mortem although it was not possible to decide whether this finding was related to the treatment. The patient with multiple myeloma showed no evidence of any effect of this agent on tumor.

Following initial chill and fever, a prolonged period of hypotension, some-

times with anuria and cardiac decompensation, occurred. Some of these reactions may have been a consequence of rapid absorption of products of destruction of tumor tissue.

The two patients showing symptomatic relief also showed chemical evidence of rapid breakdown of nitrogenous substances. This fact suggested that tissue protein, including nucleoprotein, might have been destroyed.

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DEGRADATION OF CYSTINE PEPTIDES BY TISSUES. I. EXOCYSTINE DESULFURASE AND DEHYDROPEPTIDASE IN RAT-LIVER EXTRACTS[1]

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INTRODUCTION

In 1939 Fromageot, Wookey, and Chaix (*1*) described an enzyme in liver which degraded cysteine with the formation of hydrogen sulfide and to which they gave the name "desulfurase." Subsequently Smythe (*2*) made an extensive study of this enzyme, in which he showed (1) that both cystine and cysteine were nearly equally degraded, whether under aerobic or under anaerobic conditions, and (2) that both substrates under these conditions yielded not only hydrogen sulfide but ammonia and pyruvic acid as well.

A later study of the enzyme in rat and in mouse livers confirmed Smythe's findings in all essential respects and further revealed that the hepatomas derived from these tissues were completely lacking in this enzyme, i. e., in the course of the cancerous change the erstwhile liver cells lost the enzyme (*3*, *4*, *5*).[2] In connection with this investigation the cystine peptides, *l*-cystinyldiglycine and diglycyl-*l*-cystine, were also employed as substrates. The surprising result was that not only was hydrogen sulfide evolved from liver digests containing these peptides but also the proportion of ammonia formed was very nearly the same as that derived from free cystine under the same conditions.

The first explanation that comes to mind for these results on the cystine peptides is that dipeptidase in the liver extracts effects the hydrolysis of the peptides, and the free cystine and glycine are degraded as usual; since glycine under these conditions is little if at all deaminated (*6*), practically all the ammonia found in such digests must have been derived from the cystine moiety of the peptides.[3] Accordingly, diglycyl-*l*-cystine must have been hydrolyzed at the peptide bond prior to deamination of the cystine moiety, for the amino group of the latter, which presumably furnishes the ammonia found, is linked with the carbonyl group of the glycine in the peptide. The objection to this simple explanation of a preliminary hydrolysis of the amide bond in the cystine peptides is based upon the fact that the amount of ammonia noted in the liver digests of these substrates corresponds to at least a 40-percent degradation of the cystine moiety within 2 hours of incubation, a value close to that found for free cystine under the same conditions. This would imply the presence of a very active dipeptidase in the liver extracts. The magnitude of this activity can only be guessed when it is realized that it requires more than 2 hours for extracts of the intestinal mucosa, the richest known source of animal dipeptidase, to hydrolyze the cystine peptides to this extent (*7*). It is known, moreover, from the data of Maver et al. (*8*) that the dipeptidase activity of rat liver is extremely low. If the peptides are not hydrolyzed to the free amino acids prior to the deamination, it may be conceived that the ammonia found in digests of diglycylcystine, for example, is derived from the glycyl residue of the peptide while the latter is yet intact. However, glycylglycine is little if at all deaminated under the conditions employed (*6*), and no amino acid is known to be deaminated while in peptide linkage.

It seems highly probable that the peptide bond in the peptides is hydrolyzed prior to the giving off of ammonia

[1] Delivered before the Washington, D. C., branch of the American Chemical Society, Oct. 12, 1944.

[2] The enzyme that degrades cystine is the only intracellular biocatalyst so far observed that is absent from all tumors studied (*3*, *4*). However, since in all the normal tissues studied it is present only in liver, kidney, and pancreas, it would not be fair to assume that its universal absence in neoplasms is an exclusive property of the latter sort of tissues (*5*).

[3] The dipeptidase and aminopolypeptidase present in extracts of intestinal mucosa effect the hydrolysis of cystine from *l*-cystinyldiglycine and *l*-cystinyldidiglycine, respectively, the liberated cystine crystallizing in the form of roulettes or prisms of hexagons (*7*). Intestinal mucosa does not contain the cystine-degrading enzyme (*4*).

by the substrates, and although this point could be definitely settled by employing isotopic nitrogen in the cystine moiety of diglycylcystine, two simpler kinds of proof have been attempted. One of these has been to examine the digests of the peptides for the presence of pyruvic acid, which could only have been derived from the cystine moiety of the peptides. The other has been to employ dichloracetyl-*l*-cystine as a substrate. Any evolved ammonia observed in digests of this substrate would serve at least two purposes, namely, (1) absolute proof that the peptide bond was split since the only nitrogen in this substrate is held in the bond, and (2) strong evidence that the agency which split this bond was not what is conventionally called dipeptidase.[4] The hydrolysis of the peptide bond in dichloracetylcystine might be expected to be effected by carboxypeptidase; however, there is not only very little of this enzyme in liver but it is known that this enzyme does not hydrolyze the peptide bond in either diglycylcystine or cystinyldiglycine (*7*, *9*).[5]

It is evident that the behavior of the cystine peptides under these conditions poses many interesting problems, not the least of which is the nature of the agency which splits the peptide bond in these molecules. If this agency is neither dipeptidase nor carboxypeptidase, what might it be? A possible answer lies in ideas expressed several years ago by Bergmann, Schmitt, and Miekeley (*10*), who suggested that amino acids while yet in peptide combination might be biologically dehydrogenated at the α, β position, forming the so-called dehydropeptides. Bergmann and his co-workers synthesized a number of such dehydropeptides, and the idea that such substances were actually biologic intermediates received support through the discovery of an enzyme by Bergmann and Schleich (*11*) in swine kidney, which was distinct from the known peptidases and which caused the splitting of such a substrate as glycyldehydrophenylalanine into glycine, ammonia, and phenylpyruvic acid. The designation given to this enzyme was thus dehydropeptidase. Bergmann made no speculations as to what the possible precursor or precursors of the dehydropeptides might be. It occurred to us that the cystine peptides might serve in this capacity. The desulfuration of cystine while in peptide combination could leave as residue a dehydropeptide. An obvious test of this possibility would consist in ascertaining whether liver extract contains a dehydropeptidase capable of yielding, from dehydropeptides derivable from the cystine peptides, at least the same amount of ammonia and pyruvic acid within the same digestion period as the cystine peptides yield. The presence of such an enzyme in liver has not previously been sought. We employed as substrates for this purpose chloracetyldehydroalanine and glycyldehydroalanine which may be considered to be the unsaturated, desulfurated analogs, respectively, of dichloracetylcystine and of diglycylcystine. Another test would be based upon the finding of Bergmann and Schleich that if the dehydrogenated amino acid occupies the middle position of a tripeptide no enzymatic reaction occurs; according to this, glutathione should be nearly entirely resistant to the action of the liver extracts. Since these possibilities suggest new and basic phenomena, they were investigated by using rat liver as the enzyme source and a wide variety of cystine peptides and related nonsulfur compounds as substrates.

[4] The substrate for dipeptidase must contain a free α-amino group (*23*), which is obviously lacking in dichloracetylcystine.

[5] It has been demonstrated that under conditions whereby most of chloracetyl-*l*-tyrosine is split by a carboxypeptidase preparation, cystinyldiglycine and diglycylcystine are completely unaffected (*7*, *9*). In all peptides except those with aromatic rings, the presence of a free α-amino group confers a resistance against the action of carboxypeptidase (*24*).

EXPERIMENTAL PROCEDURE

SOURCE OF ENZYME

Rats of the Buffalo and the Osborne-Mendel stock were used. No differences were observed between these stocks

or between males and females. All animals were fed Purina dog chow and water ad libitum. The rats were decapitated; the livers were immediately removed, ground with sand in a glass mortar, and suspended in three times their weight of distilled water. The suspensions were allowed to stand 1 hour at room temperature (23°–26° C.) with occasional stirring and were then centrifuged at 1,500 r. p. m. for 5 minutes. The supernatant solutions were used as the source of enzyme.

SUBSTRATES

The *l*-cystine used was a product of the Eastman Kodak Co. It was crystallized twice, and its analysis and rotation were checked. The *dl*-cystine was a Hoffmann La Roche preparation. The *l*-cysteine hydrochloric acid was also a Hoffmann La Roche product, and its mercaptan titer was checked by titration in neutral solution against porphyrindin (*12*). Glutathione was obtained from Eimer and Amend, and its mercaptan titer was likewise checked against porphyrindin. S-Benzylcysteine was prepared from cysteine and benzylbromide in the usual manner and was crystallized twice before being used; its nitrogen content was checked by analysis. Dichloracetyl-*l*-cystine was synthesized according to the method of Fischer and Suzuki (*13*),[6] was crystallized twice before being used, and its nitrogen content and melting point were checked. The melting point, determined by Dr. Hartwell, of this Institute, was 134.8°–136.8° C. (corrected).

The preparations of the cystine peptides and their derivatives, *l*-cystinyldiglycine (*14*), *l*-cystinyldidiglycine (*7*), diglycyl-*l*-cystine (*9*), *l*-cystinyl-*l*-cystine (*15*), bisanhydro-*l*-cystinyl-*l*-cystine (*16*), *l*-cysteinyl-*l*-cysteine (*15*), and anhydro-*l*-cysteinyl-*l* cysteine (*16*) have been in the possession of one of us for some time and with the exception of the first-mentioned peptide, which was synthesized according to the method of Loring and du Vigneaud (*14*), are the original preparations on which the elemental analyses were reported and in the cases of cystinyldiglycine, cystinyldidiglycine, and diglycylcystine are also the preparations on which extensive physicochemical measurements (*17*, *18*) and peptidase studies (*7*) have been performed.[7] All of these peptides and their anhydro derivatives (diketopiperazines) are crystalline. Complete descriptions of these substrates are given in the references cited, but it may be mentioned that cystinylcystine and bisanhydrocystinylcystine exist in the form of dimers (*15*, *16*).

dl-Alanine, *dl*-serine, *dl*-alanylglycine, and glycylglycine were Hoffmann LaRoche products. Glycine was an ammonia-free preparation from the Eastman Kodak Co. Glycyl-*dl*-alanine was synthesized by the method of Fischer (*19*) and was recrystallized twice before use; chloracetylalanine is an intermediate in the synthesis of this peptide.[8] Triglycine, prepared by the carbobenzoxy method, and chloracetyl-*l*-tyrosine were synthesized by one of us some years ago. Chloracetyldehydroalanine was synthesized by the method of Bergmann and Grafe (*20*).[9]

[6] The procedure of Fischer and Suzuki was slightly modified by employing potassium instead of sodium hydroxide and by decolorizing the acidified mixture with Norit prior to the extraction of the chloracetyl peptide with ether. The crude product was purified by washing with ethyl acetate, followed by solution in hot ethyl acetate, and by the addition of a mixture of dry ether and petroleum ether to incipient turbidity. The product crystallized on cooling in long sheaves of prisms.

[7] The X-ray structure of crystals of bisanhydrocystinylcystine has been described (*23*).

[8] The procedure of Fischer for the synthesis of chloracetylalanine was modified by using potassium instead of sodium hydroxide and by shaking out the acidified mixture four times with ether. The combined ether extracts were dried with anhydrous sodium sulfate and evaporated to dryness in the absence of moisture. The residual oil crystallized rapidly on cooling and the product was recrystallized from acetone-chloroform.

[9] We have found it necessary to adhere closely to the procedure described by Bergmann and Grafe and to use as starting materials pyruvic acid and chloracetamide of the highest purity. The latter compound was prepared according to the directions described in Organic Syntheses (*26*). The condensation product must be thoroughly washed with water, or unaltered chloracetamide will show up, admixed with the chloracetyldehydroalanine in the final product. With care, there is no difficulty in obtaining the crystalline product in good yield, with the proper melting point and elemental analysis, together with the property of instantaneously decolorizing permanganate in soda solution.

The glycyldehydroalanine was easily prepared from the chloracetyl compound by treatment with ammonia (*20*). These dehydropeptides are entirely stable in aqueous solution. Analyses and melting points were carefully checked.

DIGESTION MIXTURES

The substrate solutions were made up so that the concentration per cubic centimeter of the nonsulfur compounds was 25×10^{-6} mols, and the concentration per cubic centimeter of the sulfur-containing compounds was 25×10^{-6} mols sulfur. Racemic compounds were made up in double this concentration. All substrates except cystine, cystinylcystine, and S-benzylcysteine were dissolved in distilled water. The first two substances were dissolved in dilute alkali, an equivalent amount of acid was added rapidly, and the necessary aliquots were removed quickly before crystallization occurred. The S-benzylcysteine, which was treated in the same way and which precipitated under these conditions immediately, was transferred to the digest in nearly uniform aliquots of the fresh suspension. At the concentration used, bisanhydrocystinylcystine is sufficiently soluble in distilled water (*16*). The stock solutions of the chloracetyl peptides were neutralized before use.

The digestion mixtures were prepared by mixing 2 cc. of the liver extract with 1 cc. of phosphate buffer at pH 7.0 and 1 cc. of substrate solution. The final pH of the mixture was 6.8–6.9. The controls consisted of mixtures similar to the foregoing only containing (1) 2 cc. of water in place of the liver extract, and (2) 1 cc. of water in place of the substrate solution. The mixtures were placed in the incubator at 37° C. for stated periods. Ammonia was estimated by alkalizing the digest with 1 cc. of saturated potassium carbonate (plus a drop of caprylic alcohol) and by aerating the mass for 30 minutes into a sulfuric acid trap. The ammonia was determined in the trap by nesslerization.[10] The controls under (1) were invariably negative, i. e., the substrates alone, under the conditions employed, yielded no measurable quantities of ammonia. The liver extracts alone (control 2) gave appreciable ammonia values, and these were subtracted from those found in the text mixtures. All runs were made in duplicate, and each substrate was tested with livers from several different animals. Duplicate experiments (on the same liver) agreed within 5 percent. Data for the same substrate with livers of different animals agreed within 15 percent. The ammonia determinations were made on the digests under aerobic and under anaerobic conditions. Little if any difference in the results was obtained (*2*, *3*).

Pyruvic acid was estimated in the designated digests as the 2:4 dinitrophenylhydrazone (*21*, *22*). For these determinations, the amount of the digestion mixtures was increased tenfold proportionately. Blanks on the extracts were practically negligible. A B Wratten No. 58 green filter was used for the readings.

For the detection of the appreciable quantities of ammonia (based on more than 30 individual experiments on cystine alone) and of pyruvic acid, it has not been found necessary to use the chloroform purification of the extracts recommended by Smythe (*2*).

Conventional dipeptidase and aminopolypeptidase activity was measured in the liver extracts by using *dl*-alanylglycine for the former, and triglycine for the latter, and by titrating with alkali in 85 percent alcohol to a blue color with thymolphthalein. Similarly, carboxypeptidase activity was estimated with chloracetyl-*l*-tyrosine as substrate.

The pH optima of these peptidases are higher than the optimum employed (ca. pH 7.0), but it was considered desirable to ascertain the activity of these enzymes under the same conditions as

[10] The ammonia readings must be made directly after adding the Nessler reagent. Otherwise, on standing some cloudiness because of the entrapped caprylic alcohol will ruin the reading (27).

those involved in the degradation of the cystine peptides.

RESULTS

The data on the sulfur-containing substrates are given in table 1. With one exception, the peptides of cystine and cysteine are as readily degraded to ammonia, pyruvic acid, hydrogen sulfide, etc., as cystine or cysteine alone. The exception is glutathione, whether reduced or oxidized. In this tripeptide the sulfur-containing amino acid is in the middle of the chain, and its amino and carboxyl groups are tied off in peptide linkage with glutamic acid and with glycine, respectively. For the degradation of the cystine or cysteine in the peptides, either the amino or the carboxyl group must apparently be free. This statement is supported by the failure of the diketopiperazines, anhydrocysteinylcysteine and bisanhydrocystinylcystine, which like glutathione have neither a free amino nor a free carboxyl group on the cystine or cysteine moiety, to be degraded under conditions whereby cystine peptides with a free group on the moities are readily degraded.

Cystine or cysteine in order to be degraded must have not only a free amino or a free carboxyl group but also the sulfur group must be free. Substitution of the sulfur as in S-benzylcysteine renders the molecule resistant to enzymatic action.[11]

The case of dichloracetylcystine is of particular interest. Diglycylcystine readily yields ammonia under the experimental conditions chosen, and it is most probable that this ammonia comes from the cystine moiety of the peptide and not from the glycine. (See tabulation on p. 215). Glycine and glycylglycine yield no ammonia under these conditions. This is, however, no unequivocal proof that the ammonia derived from the liver digests of the diglycylcystine actually came from the cystine. In dichloracetylcystine, on the other hand, there are only two nitrogen atoms in the molecule, and these belong to cystine. Moreover, these nitrogen atoms are bound in peptide linkage. The evolution of ammonia nitrogen from digests of the chloracetylpeptide indicate definitely, therefore, that not only is this nitrogen derived from the cystine part of the peptide but also that the peptide linkage must necessarily have been hydrolyzed prior to this derivation.

[11] Methionine is oxidatively deaminated to only a slight extent and does not appear to liberate mercaptan under these conditions (*3*).

TABLE 1.—*Ammonia nitrogen and pyruvic acid split from cystine, cysteine, and cystine peptides in the presence of rat-liver extracts*

Mixtures incubated 2 hours at 37° C. consisted of 2 cc. rat-liver extract + 1 cc. phosphate buffer at pH 7.0 + 1 cc. substrate solution. Concentration of substrates was 25×10^{-6} mols per cubic centimeter of levo and twice this for racemic compounds.

Substrate	Ammonia nitrogen [1] (Mols x 10^6)	Pyruvic acid [2] (Mols x 10^6)
l-Cystine [3]	11	6
dl-Cystine [3]	11	
l-Cystinyldiglycine [3]	10	3
*l*Cystinyldidiglycine [3]	11	4
Diglycyl-*l*-cystine [3]	12	6
l-Cystinyl-*l*-cystine [3]	12	5
Bisanhydro-*l*-cystinyl-*l*-cystine [4]	0	0
l-Cysteine [3]	7	7
l-Cysteinyl-*l*-cysteine [3]	6	4
Anhydro-*l*-cysteinyl-*l*-cysteine [4]	0	0
Glutathione [4]	0	0
Oxidized glutathione [4 5]	0	0
Oxidized cysteine [3 5]	9	
S-Benzylcysteine [4 6]	0	0
Dichloracetyl-*l*-cystine [3]	10	6

[1] Experiments were performed in the presence of air, but practically the same results for ammonia nitrogen are obtained under anaerobic conditions (*3*, *4*).

[2] Since the determinations were made on mixtures made up proportionately tenfold, the data given for pyruvic acid in the table represent one-tenth of the value observed, to be comparable with the ammonia values.

[3] Hydrogen sulfide present in the digests. Under both aerobic and anaerobic conditions the digest containing thiol compounds revealed the presence of more hydrogen sulfide than did the digests containing the disulfide compounds. None of the substrates alone gave off hydrogen sulfide or ammonia under the experimental conditions employed. Hydrogen sulfide was qualitatively detected by its odor.

[4] No apparent ammonia or hydrogen sulfide even after extending the incubation period to 4 hours.

[5] These substances had been oxidized to the corresponding disulfide forms by adding to the thiol forms the equivalent amount of porphyrindin (*12*). The leuco base itself yields no ammonia.

[6] The S-benzylcysteine had been added to the digestion mixtures as an aliquot of a suspension of the freshly precipitated material. Lest the negative results obtained with this material be attributed to its initial insolubility in water, digestion experiments with aliquots of freshly prepared suspensions of cystine and cystinylcystine were similarly performed. The results with these suspensions were identical with those given. Heterogeneity is no essential barrier to enzymatic action.

Pyruvic acid was isolated from the digests of those substrates tested. This product could have been derived only from the cystine moieties of the peptides (since glycine, even if it were deaminated, would not yield pyruvic acid directly) and constitutes another proof that the peptides are split at the amide linkage.

The amount of ammonia liberated by the enzymatic degradation of cystine and of the cystine peptides corresponds at best to some 40 percent of the total substrate being broken down. The amount of pyruvic acid observed corresponds to even less than this. In such mixtures, with a crude liver extract, it would be expected that these figures for ammonia and for pyruvic acid were minimal owing to the many further reactions which these products, together with the unchanged substrate and substances natural to the liver extract, are capable of yielding (*2*).

No attempt was made to measure quantitatively the hydrogen sulfide evolved, but its presence in the particular digests was unmistakable. In digests containing cysteine or cysteinylcysteine, under anaerobic conditions, the color of the digest after 2 hours of incubation was almost black from the ferrous sulfide formed, and the tubes reeked of the gas. Less darkening of the digest occurred under anaerobic conditions with cystine and the cystine peptides, but here, too, the presence of the gas was unmistakable. Under aerobic conditions, the odor of hydrogen sulfide in the digests was much fainter, being least so for cystine and for its peptides. The definitely smaller amount of gas detectable in digests of the disulfide than of the thiol compounds is very probably to be attributed to reaction of the liberated hydrogen sulfide with the residual unaltered disulfide compound whereby the latter is converted into a mercaptan compound and the gas into sulfur (*2*). This may be represented by equation 1:

$$RSSR + H_2S \rightarrow 2\,RSH + S \qquad (1)$$

The unchanged disulfide thus blots up the evolved hydrogen sulfide as fast as it is formed. In the case of the thiol-substrates, such as cysteine and cysteinylcysteine, no such reaction can occur, and the hydrogen sulfide may then accumulate. The smaller amount of gas detectable under aerobic than under anaerobic conditions may be attributed to autoxidation. No trace of hydrogen sulfide could be detected in controls of either substrate or of liver extract.

The amount of ammonia liberated from the thiol compounds appears to be generally less than that liberated from the disulfide substrates, but the significance of this difference, if it is significant, is not apparent at the present time. It is possible that this difference may be due to secondary reactions entirely, and it would be better to emphasize the fact that the ammonia split from thiol and from disulfide compounds under the same conditions is in both cases rather of the same order of magnitude.

The rate of the evolution of ammonia from digests of cystine and of the cystine peptides is described in table 2. It is evident that this rate is very nearly the same for all the substances tested. It is of some interest that diglycylcystine and dichloracetylcystine should be degraded apparently as rapidly as cystine, for it implies that the peptide

TABLE 2.—*Rate of splitting of ammonia N from cystine and cystine peptides treated with rat-liver extract* [1]

Time of incubation in minutes	Ammonia N split off from—				
	Cystine (Mols x 10^6)	Cystinyldiglycine (Mols x 10^6)	Cystinyldiglycine (Mols x 10^6)	Diglycylcystine (Mols x 10^6)	Dichloracetyl cystine (Mols x 10^6)
30	4	4	6	4	-------
60	6	7	6	7	7
90	10	9	-------	10	-------
120	13	12	11	13	14
150	15	15	-------	15	-------
180	15	16	15	16	18

[1] For experimental conditions, see headnote to table 1.

bond in the two peptides must be extremely rapidly hydrolyzed. The methods of measurement of the rate of ammonia evolution from these compounds are hardly sensitive enough to detect the possible time lag involved in the splitting of the peptide bond.

The data on nonsulfur compounds related to the substrates described in tables 1 and 2 are given in the following tabulation. Cystine is a β substituted alanine, and thus alanine and its peptides may be considered to be sulfurless homologues of cystine and its peptides. Serine was studied because this substance has the same carbon chain as cysteine, with oxygen on the β carbon in place of sulfur. Chloracetyl-*l*-tyrosine was included, for it was thought of interest to observe the behavior of a peptide containing an amino acid with another kind of polar grouping. It is evident, however, that with the exception of *dl*-alanine, none of the substances described in the tabulation yield ammonia under the digestion conditions noted, and in the case of alanine the deamination is presumably the result of the action of *d*-amino acid oxidase.

Ammonia N split from amino acids and peptides treated with rat-liver extract [1]

Substrate	*Ammonia N (Mols x 10^6)*
dl-Alanine	2
dl-Serine	0
dl-Alanylglycine	0
Glycyl-*dl*-alanine	0
Glycine	0
Glycylglycine	0
Chloracetyl-*dl*-alanine	0
Chloracetyl-*l*-tyrosine	0
l-Tyrosine	0
Triglycine	0
Chloracetamide	0

[1] Tested aerobically. No pyruvic acid observed.

In table 3 are given data for the conventional representation of dipeptidase, aminopolypeptidase, and carboxypeptidase activity, using respectively, alanylglycine, triglycine, and chloracetyltyrosine as substrates. It is clear that under the conditions studied, including a pH of 6.8–7.0, there is little if any observable activity of these enzymes. The low value of dipeptidase activity in rat liver with *dl*-leucylglycine as substrate, was pointed out earlier (*8*). The data on alanylglycine and on glycylalanine in the tabulation further confirm the almost complete absence of dipeptidase activity in the digests; for were this enzyme present to any fair degree, the substrates mentioned would have been hydrolyzed, and the liberated alanine would presumably have yielded ammonia. The absence of carboxypeptidase in the liver extracts is similarly confirmed by the absence of ammonia from digests containing chloracetylalanine (tabulation).

TABLE 3.—*Dipeptidase, aminopolypeptidase, and carboxypeptidase activity in rat-liver extracts*

[2cc. rat liver extract + 1 cc. phosphate buffer at pH 7.0 + 1 cc. substrate solution containing 50 x 10^{-6} mols of *dl*-alanylglycine or 25 x 10^{-6} mols of either triglycine or chloracetyl-*l*-tyrosine and incubated at 37° C. 2 hours. Mixtures then treated with alcohol to 85 percent and titrated with 0.01N alcoholic potassium hydroxide and thymolphthalein. Blanks were subtracted from the test readings.]

Substrate	0.01N potassium hydroxide consumed [1]	Hydrolysis
	Cubic centimeters	*Percent*
dl-Alanylglycine	0.1	(4)
Triglycine	0	0
Chloracetyl-*l*-tyrosine	0	0

[1] 2.5 cc. corresponds to 100-percent splitting of chloracetyl-*l*-tyrosine and of the optically active form of *dl*-alanylglycine; complete splitting of both bonds in triglycine requires 5 cc. of the alkali.

The course of ammonia production in digests of rat-liver extracts with the dehydropeptides and the presence in such digests of pyruvic acid are described in table 4. Chloracetyldehydroalanine and glycyldehydroalanine may be considered to be the desulfurated analogs, respectively, of dicholoracetylcystine and of diglycylcystine.

The data reveal the presence of powerful dehydropeptidase activity in rat-liver extracts. The enzyme apparently acts equally and progressively on chloracetyldehydroalanine and glycyldehydroalanine. Ammonia and pyruvic acid appear in appreciable quanti-

TABLE 4.—*Ammonia and pyruvic acid formed in digests of rat-liver extracts with dehydropeptides*[1]

Time of incubation in minutes	Chloracetyldehydroalanine		Glycyldehydroalanine	
	Ammonia (Mols x 10^6)	Pyruvic acid (Mols x 10^6)	Ammonia (Mols x 10^6)	Pyruvic acid (Mols x 10^6)
30	6	.....	5	.....
60	10	.....	9	.....
90	14	.....	14	.....
120	19	16	21	19

[1] For experimental conditions, see headnote to table 1.

ties in digests of these substrates. The rate of appearance of the ammonia in such digests is somewhat greater than in digests containing equivalent amounts of the corresponding cystine peptides. After 2 hours of incubation the ammonia found in digests of the dehydropeptides corresponds to a degradation of these subtrates of about 80 percent as contrasted with about 40 percent when the cystine peptides are used as substrates.[12] However, the digests of the cystine peptides also contain hydrogen sulfide and sulfur as products of the enzymatic reaction, and the possible effects of these products on the activity of the enzyme or enzymes concerned are not known.

The rate of degradation of diglycylcystine and dichloracetylcystine to yield ammonia and pyruvic acid is very nearly the same, and likewise the rate of degradation of glycyldehydroalanine and chloracetyldehydroalanine to yield these same products is also very nearly the same. The presence or absence of an amino group in either group of substrates is apparently a matter of indifference to the enzymatic processes concerned. Like the case of dichloracetylcystine, the ammonia which appears in digests of chloracetyldehydroalanine could only have appeared subsequent to the rupture of the peptide bond.

The pH optimum for the activity of dehydropeptidase, like that of the peptidases for saturated peptides (dipeptidase, etc.), is close to 8 (*11*). The high activity of the rat-liver dehydropeptidase is further emphasized when it is recalled that the working pH of the studies reported in this paper was slightly below 7. The presence of cystine or cysteine (and presumably of their peptides) has no effect on the activity of the dehydropeptidase (*11*).

In order to see whether the effect of the rat-liver extracts on the cystine peptides and on the dehydropeptides could be separated, dialysis studies of such extracts were undertaken. It was found that after a 24-hour period of dialysis of these extracts in cellophane bags against either running tap water or a large volume of distilled water, the ability of the extracts to degrade both cystine and the cystine peptides was completely lost whereas the ability to degrade the dehydropeptides was completely retained. A clean-cut separation can thus be easily attained. These dialysis studies were repeated many times, each individual test in duplicate, and the livers of eight rats were employed; the findings were entirely reproducible. The extracts to be dialyzed were prepared as stated previously and were divided in two. One half was dialyzed, and the other half was placed in a rubber-stoppered bottle suspended in the water container outside the bag. In this way, the control extract was kept at the same temperature for the same length of time as the dialyzed portion. The activity of such control extracts against both cystine peptides and dehydropeptides was little different from that of freshly prepared extracts. At the end of the dialysis period, the extract inside the cellophane bag contained an amount of precipitated protein. The activity of the dialyzed extract toward the dehydropeptides and the lack of activity toward the cystine peptides were the same

[12] The 2 cc. of rat liver extract used as the source of enzyme in each determination contains almost invariably 0.06–0.08 mg. of ammonia nitrogen. It is obvious that the ammonia nitrogen in the digests which is made up of this quantity plus that derived by enzymatic action on the substrates can be estimated with ease and with accuracy. The total nitrogen in such digests may be anywhere between one and a half to three or four times that of the extract control. The substrate controls never yield more than an unmeasurable trace of ammonia.

whether the entire contents of the bag or only the supernatant centrifuged from the precipitated material was used. The problem of the mechanism of the loss of the cystine-degrading system by dialysis (presence of dialyzable coenzyme factors, etc.) is deferred for future study. The data for the present dialysis experiments are given in table 5.

TABLE 5.—*The effect of dialysis of rat-liver extracts*[1] *on the enzymatic activity*

Substrate	Activity of extracts		
	Control (Mols NH_3-N x 10^6)	Dialyzed (tap water) (Mols NH_3-N x 10^6)	Dialyzed (distilled water) (Mols NH_3-N x 10^6)
l-Cystine	9	5	5
Diglycyl-*l*-cystine	10	5	5
Dichloracetyl-*l*-cystine	9	5	5
Glycyldehydroalanine	18	17	18
Chloracetyldehydroalanine	16	17	17

[1] Extracts prepared as described and dialyzed 24 hours in cellophane bags against either running tap water or distilled water. Control extracts were aliquots of same extracts dialyzed and were kept under identical conditions. Incubation mixtures were same as those described in tables 1 and 4. Incubation time 2 hours at 37° C.

[2] Both enzymes may be inactivated by heating the extract at 90° C. for 5 minutes.

[3] 18°–20° C.

[4] 24°–26° C.

[5] No evidence of hydrogen sulfide.

DISCUSSION

Rat liver possesses a powerful enzymatic system which rapidly degrades peptides of cystine with the production of hydrogen sulfide, ammonia, and pyruvic acid.[13] The sulfide and the pyruvic acid could only have been obtained from the cystine moiety of the peptides while the presence of ammonia in digests of dichloracetylcystine proves that this product also is derived from the cystine portion. Not only dipeptides but also tripeptides containing cystine are degraded, but in the latter molecules cystine must apparently occupy one of the end positions in the chain. Thus, although cystinyldidiglycine is as rapidly degraded as is cystine, glutathione is apparently unaffected (c. f. footnote 13). Cystine (or cysteine) placed in the middle of a tripeptide, or cystine (or cysteine) bound by self-condensation in a diketopiperazine ring, possesses neither a free amino nor a free carboxyl group and is inert toward the liver enzyme system (table 1). Cystinyldiglycine, cystinyldidiglycine, diglycylcystine, and dichloracetylcystine are degraded at nearly equal rates: in all these peptides, the cystine moiety possesses either a free α-mino or a free α-carboxyl group. We may therefore designate the system which degrades the peptides of cystine as an exo-"ase."

The problem was raised in the introduction whether (1) the peptides of cystine are hydrolyzed to free cystine and glycine (glycylglycine) by a peptidase in the liver extract and the cystine subsequently degraded, or whether (2) the exocystine moiety of the intact peptide is first desulfurated to the corresponding dehydropeptide and the latter then hydrolyzed by a dehydropeptidase to products which include ammonia and pyruvic acid. That the peptide bond is certainly split is evident from the presence of pyruvic acid in all the digests of the susceptible peptides and of ammonia in digests of dichloracetylcystine (table 1). Alternative 1 is quite unlikely because of the following reasons: the practical absence under the experimental conditions of dipeptidase, aminopolypeptidase, and carboxypeptidase activity (table 3), and the absence of any effect of the liver extracts on the nonsulfur-containing peptides, analogous to the cystine peptides, listed in the tabulation on p. 215. The validity of alternative 1 would demand, therefore, the presence in rat extracts of a powerful peptidase effective on cystine peptides but ineffective toward such classic substrates as leucylglycine, alanylglycine, triglycine, or chloracetyltyrosine. While the existence of such a peptidase cannot be arbitrarily

[13] Peptides of cysteine, as well as cysteine, are also degraded by the liver system (table 1), but to avoid needless repetition only the word "cystine" will be employed in the discussion, with the understanding that so far as we know all conclusions applicable to the behavior of cystine peptides hold likewise for cysteine peptides.

denied, there is not sufficient evidence to postulate the presence of a unique cystine peptidase which splits cystine peptides so rapidly that the liberated cystine can be degraded at a rate close to that of free cystine alone (table 2).

On the other hand, the new observation of a very active dehydropeptidase in the liver extracts inclines us to the acceptance of alternative 2. This enzyme produces an 80 percent degradation of chloracetyl (or glycyl) dehydroalanine to ammonia and pyruvic acid in 2 hours of incubation and under the same experimental conditions as those employed when the cystine peptides yielded these same products (table 4). An important fact is that the substrate specificity of this dehydropeptidase parallels that of the cystine peptide-degrading system, for the former like the latter attacks chloracetyl as readily as glycyl peptides (tables 2 and 4), and both systems also act only on exopeptides. Thus while both cystinyldidiglycine and diglycyldehydrophenylalanine are enzymatically hydrolyzed to yield among other products ammonia and pyruvic acid, glutathione (oxidized or reduced) and glycyldehydrophenylalanylglycine are not at all affected (table 1 and (*11*)).[14]

It is therefore possible tentatively to conceive of the degradation of the cystine peptides by the liver extracts as occurring in three successive stages: (1) An enzymatic desulfuration of the intact exopeptide to yield hydrogen sulfide (and sulfur) and the corresponding dehydropeptide; (2) an enzymatic hydrolysis of the dehydropeptide to aminoacrylic acid; and (3) the nonenzymatic, spontaneous hydrolysis of the unstable aminoacrylic acid to pyruvic acid and ammonia. These reactions may be formulated as follows, by choosing dichloracetylcystine for purposes of illustration as substrate, although analogous formulations could be given for all the susceptible cystine peptides employed:[15]

1. $[ClCH_2CONHCH(COOH)CH_2S-]_2 \longrightarrow$
   Dichloracetylcystine
   $2\,ClCH_2CONHC(COOH)=CH_2 \longleftarrow + H_2S + S$
   Chloracetyldehydroalanine

2. $ClCH_2CONHC(COOH)=CH_2 \xrightarrow{H_2O}$
   $ClCH_2COOH + NH_2C(COOH)=CH_2$
   Aminoacrylic acid

3. $NH_2C(COOH)=CH_2 \xrightarrow{H_2O} NH_3 + CH_3COCOOH$

The degradation of free cystine alone may thus consist successively in an enzymatic desulfuration to aminoacrylic acid, followed by the spontaneous reaction 3.[16]

Evidence that the desulfuration reaction 1 precedes the hydrolyses (2 and 3) may be derived on the basis of the behavior of S-benzylcysteine (table 1). This compound has all the obvious structural characteristics for susceptibility to degradation, i. e., chain length and free amino and carboxyl groups, except that the sulfur is blocked. Apparently when the possibility of desulfuration is removed, so too is the possibility of any further degradation under the conditions employed. While the desulfuration must be the first point of attack on the free cystine molecule, it may be assumed by analogy that the same sequence of events would hold in the case of cystine bound in the peptides.

The dialysis experiment (table 5) indicates clearly that the enzyme responsible for the degradation of cystine and presumably for the desulfuration of the cystine peptides (reaction 1), and

[14] The γ bond in glutathione might be expected to provide a point of resistance of this enzyme toward a possible enzymatic hydrolysis of the bond, but it does not suggest any reason per se why the cysteine moiety is not desulfurated.

[15] Cystinyl peptides with the cystine in acyl position such as cystinyldiglycine or cystinyldidiglycine by desulfuration might be expected to yield dehydroalanylglycine or dehydroalanylglycylglycine, respectively. Such free dehydropeptides have not yet been synthesized, but there is no reason to suppose that they would not be hydrolyzed by dehydropeptidase. Indeed, if reactions 1–3 are valid for cystine peptides in which cystine occupies the terminal position, the fact that the peptides in which cystine occupies the acyl position yield the same products at the same rate is evidence that the process of degradation is the same for both kinds of peptides. Recently, Doherty, Tietzman, and Bergmann (*28*) synthesized a number of N-acyldehydropeptides and anhydrides.

[16] The presence of both hydrogen sulfide and of sulfur in digests of rat-liver extracts with cystine was shown by Smythe (*2*).

the enzyme responsible for the hydrolysis of the dehydropeptides are readily distinguishable factors. The former is inactivated by dialysis, while the latter is unaffected. Of equal or greater interest would be the discovery of a method of inactivating the dehydropeptidase while leaving the desulfuration mechanism intact, and further investigations to test this possibility are in progress.

The degradation of cystine or of its peptides is not only a very active process, but it is also nonoxidative. It is thus not only a more powerful system than that of the oxidative deamination of amino acids, but it is also qualitatively entirely different.

The complete degradation of the cystine peptides to the products noted (reactions 1–3) involves the rupture of at least four different kinds of bonds. These are: by reaction 1, the splitting of an S–S and an S–C bond; by reaction 2, the hydrolysis of a substituted amide bond; and by reaction 3, the hydrolytic splitting of an N–C bond.[17]

The data yield two new observations. One is the very strong probability that in the degradation of the cystine peptides we have the first authentic case of an amino acid which is attacked while it is yet in peptide linkage. The second is the fact of the presence of a very active dehydropeptidase in liver. It is felt that the evidence presented tends to support the suggestion which Bergmann et al. advanced several years ago, namely, that amino acids may be dehydrogenated at the α, β position while yet in peptide combination (*10*). Through the mechanism of desulfuration, the exocystine peptides may be considered as at least one of the possible types of substrates for this purpose.

In view of the structural requirements for the cystine peptide substrates in the desulfuration mechanism, it is suggested that the name given to the system involved in reaction 1 be "exocystine desulfurase." The designation, dehydropeptidase, which was coined by Bergmann and Schleich (*11*), is sufficient to describe the system responsible for reaction 2.

[17] The dehydropeptides may possess in equilibrium with the acrylic acid form the following isomeric formula (*20*):

$$RCH_2CON=C(CH_3)COOH$$

SUMMARY

Not only cystine but also peptides of cystine have been found to be rapidly degraded under similar conditions at nearly the same rate by rat-liver extracts with the production of hydrogen sulfide, pyruvic acid, and ammonia. The susceptible peptides comprise: *l*-cystinyldiglycine, *l*-cystinyldidiglycine, diglycyl-*l*-cystine, dichloracetyl-*l*-cystine, *l*-cystinyl-*l*-cystine, and *l*-cysteinyl-*l*-cysteine. For susceptibility, the cystine in the peptide must be on either end of the chain and hence must have either a free α-amino or a free α-carboxyl group. Glutathione, whether oxidized or reduced, and the diketopiperazines of *l*-cystine or of *l*-cysteine are not affected. S-benzylcysteine is also resistant, and this behavior suggests that desulfuration precedes any further changes in the molecule.

The presence of pyruvic acid in digests of all the susceptible peptides, and particularly of ammonia in digests containing dichloracetylcystine, indicates that the peptide bond of the substrates was hydrolyzed. But rat-liver extracts under the conditions employed possess little or no dipeptidase, aminopolypeptidase, or carboxypeptidase activity. These extracts, however, were found to contain a highly active dehydropeptidase, capable of splitting chloracetyldehydroalanine or glycyldehydroalanine to 80 percent in 2 hours of incubation with the production of pyruvic acid and ammonia.

Dialysis of the liver extracts for 24 hours resulted in the complete disappearance from the extracts of the ability to degrade cystine or the cystine peptides while the ability to degrade the dehydropeptides was completely retained.

In the light of these findings and of the suggestive similarities in the struc-

tural specificity of the cystine peptide degrading system and of dehydropeptidase, it is suggested that the degradation of the cystine peptides under the conditions noted is produced successively by (1) an enzymatic desulfuration of the peptides by an agency which we have called "exocystine desulfurase," yielding besides hydrogen sulfide (and sulfur) the corresponding α, β unsaturated dehydropeptide, followed by (2) the enzymatic hydrolysis of the dehydropeptide by the highly active dehydropeptidase to aminoacrylic acid, which finally leads to (3) the spontaneous hydrolysis of the unstable aminoacrylic acid to pyruvic acid and ammonia.

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DEGRADATION OF CYSTINE PEPTIDES BY TISSUES. II. DISTRIBUTION OF EXOCYSTINE DESULFURASE AND DEHYDROPEPTIDASE IN TISSUE EXTRACTS OF VARIOUS SPECIES[1]

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In the previous paper in this series, we suggested that the degradation by rat-liver extracts of cystine peptides, in which cystine occupies either of the end positions (exopeptides), proceeds according to the following scheme:

1. Exocystine peptides → Dehydropeptides + hydrogen sulfide + sulfur
2. Dehydropeptides → α-Aminoacrylic acid + other members of the peptide chain
3. α-Aminoacrylic acid → Pyruvic acid + ammonia

Step 1 consists in an enzymatic desulfuration of the cystine moiety of the peptides by exocystine desulfurase to yield the α, β dehydropeptides (peptides of α-aminoacrylic acid). Step 2 consists in an enzymatic hydrolysis of the dehydropeptides by dehydropeptidase to yield aminoacrylic acid plus any other (saturated) amino acids present originally in the peptide chain. Step 3 involves finally the spontaneous hydrolysis of the unstable aminoacrylic acid to pyruvic acid and ammonia, which, together with hydrogen sulfide and sulfur, are the end products of the specific degradation of the cystine moiety of the peptides.

Part of the evidence taken to support the concept of this sequence of events was the observation of a highly active dehydropeptidase in the rat-liver extracts.[2] This enzyme caused the hydrolysis of at least 80 percent of added dehydropeptides to pyruvic acid and ammonia within 2 hours of incubation. If the scheme given above is correct, tissues which degrade cystine peptides to products which include pyruvic acid and ammonia must also be able to degrade dehydropeptides to these products as well. The converse need not necessarily hold, i. e., tissues may be able to degrade dehydropeptides without attacking cystine peptides, for it is conceivable that dehydropeptides may arise from the biologic dehydrogenation of amino acids other than cystine, while such acids are yet in peptide linkage. It was therefore considered desirable to study the capacity of various tissue extracts derived from different species of animals to effect (1) the degradation of a cystine peptide, and (2) the degradation of a dehydropeptide.

EXPERIMENTAL PROCEDURE

The tissues, which included liver, kidney, pancreas, spleen, brain, and muscle, were derived from rats, mice, rabbits, and guinea pigs. Extracts of these tissues were prepared in identical fashion with those of the rat livers described in the preceding paper and were studied within an hour of preparation. Each tissue was thus studied under the same conditions. The substrates employed were *l*-cystine, dichloracetyl-*l*-cystine, and chloracetyldehydroalanine, and the extent of enzymatic activity was determined by the estimation of the ammonia split from these substrates after 2 hours of incubation at 37° C. The concentration of the substrates per cubic centimeter was 25×10^{-6} mols. These peptides were chosen because the only nitrogen in the molecules is in peptide linkage, and hence any ammonia observed in the digests must have been derived subsequent to the splitting of the linkage. Chloracetyldehydroalanine may also be considered the desulfurated, unsaturated analog of dichloracetylcystine.[3] The data are given in table 1.

[1] Delivered before the Washington, D. C., branch of the American Chemical Society, Oct. 12, 1944.

[2] See preceding paper in this Journal.

[3] It was shown that when rat-liver extracts were used, substantially the same results were obtained with glycyl as with chloracetyl peptides of cystine or of dehydroalanine. It is expected that this same equivalence between glycyl and chloracetyl peptides would hold in the case of other tissues.

TABLE 1.—*Enzymatic activity of the tissues of various species*

[Digestion mixtures composed of 2 cc. extract+1 cc. phosphate buffer at pH 7.0+1 cc. substrate solution containing 25 x 10^{-6} mols; control on extract determined by using 1 cc. of water in place of substrate solution. Incubation time was 2 hours at 37° C.]

Tissue	Rat tissues [1] with—			Mouse tissues [2] with—			Rabbit tissues with—			Guinea pig tissues with—		
	Cystine (Mols NH_3–N x 10^6)	Dichloracetylcystine (Mols NH_3–N x 10^6)	Chloracetyldehydroalanine (Mols NH_3–N x 10^6)	Cystine (Mols NH_3–N x 10^6)	Dichloracetylcystine (Mols NH_3–N x 10^6)	Chloracetyldehydroalanine (Mols NH_3–N x 10^6)	Cystine (Mols NH_3–N x 10^6)	Dichloracetylcystine (Mols NH_3–N x 10^6)	Chloracetyldehydroalanine (Mols NH_3–N x 10^6)	Cystine (Mols NH_3–N x 10^6)	Dichloracetylcystine (Mols NH_3–N x 10^6)	Chloracetyldehydroalanine (Mols NH_3–N x 10^6)
Liver	10	10	20	8	8	15	4	4	12	3	2	12
Kidney	4	4	20	3	3	23	3	3	14	2	2	16
Pancreas	2	2	10	1	1	4	[3] 1	[3] 1	[3] 3	2	2	10
Spleen	0	0	0	0	0	0	0	0	0	0	0	0
Brain	0	0	0	0	0	0	0	0	0	0	0	0
Muscle [4]	0	0	0	0	0	0	0	0	0	0	0	0

[1] Osborne-Mendel stock.
[2] 40 strain A mice used.
[3] It was difficult to demarcate the rabbit pancreas from the surrounding fat, and consequently the values given should probably be somewhat higher.
[4] Skeletal.

DISCUSSION

The data in table 1 shows that within the limits set by the experimental conditions, extracts of liver, kidney, and pancreas from the species studied, prepared in the same fashion degrade both cystine peptides and dehydropeptides. On the other hand, extracts of spleen, brain, and muscle degrade neither the cystine peptides nor the dehydropeptides. It would appear that the capacity for the enzymatic degradation of the cystine peptides and the capacity for the enzymatic hydrolysis of dehydropeptides either occur together in certain tissues or do not occur at all in other tissues. These findings are in agreement with the idea that the degradation of the cystine peptides involves the consecutive action of exocystine desulfurase and dehydropeptidase.[4] Previous dialysis studies revealed that these enzymes are distinct entities. The fact that in extracts of liver, pancreas, and kidney the rate at which the dehydropeptides are split is very much greater than the rate at which the cystine peptides are degraded suggests that in the degradation of the latter substrates exocystine desulfurase is the limiting rate factor. The active tissues of the rat and the mouse exceed those of the rabbit and the guinea pig.

The activity of extracts of spleen, brain, and muscle has been described as null. This does not imply the absolute absence from such extracts of either enzyme but simply indicates that under the experimental conditions employed no evidence of enzymatic action was revealed. Since the activity of the extracts is demonstrated by the quantity of ammonia appearing in the digests, it is possible, according to the scheme outlined, that the failure to observe this product in extracts of spleen, brain, and muscle might be due to the absence of dehydropeptidase, even though the desulfurase were present. If this possibility were true, then the presence of hydrogen sulfide in these digests should be observed. The fact that no trace of the gas is present in digests of the cystine peptide with extracts of spleen, brain, and muscle indicates the absence of appreciable desulfurase activity. Moreover, the absence of dehydropeptidase would presumably not hinder the production of ammonia, etc., through the degradation of free cystine (table 1). The possibility must, of course, be considered that still other tissues not studied in these and other species may yet be found to possess either the desulfu-

[4] See preceding paper in this Journal.

rase or the dehydropeptidase but not both. We have studied only six tissues in four kinds of rodents. Until the search is considerably widened, we can only state provisionally that at the present time the evidence tends to support the mechanism proposed for the degradation of the cystine peptides whereby certain tissues possess the capacity of first desulfurating these peptides and then hydrolyzing the resulting dehydropeptides with the production, among other substances, of ammonia and pyruvic acid.

SUMMARY

A study was made of the capacity of extracts of liver, kidney, pancreas, spleen, brain, and skeletal muscle from rats, mice, rabbits, and guinea pigs to produce ammonia when incubated with *l*-cystine, dichloracetyl-*l*-cystine, and chloracetyldehydroalanine.

Extracts of the liver, kidney, and pancreas from these species yielded ammonia from all these substrates. By far the greater proportion of ammonia was observed when chloracetyldehydroalanine was used as the substrate. Extracts of the spleen, brain, and muscle yielded no evidence of this product from any of the three substrates.

It is concluded that the capacity of certain tissues to degrade cystine peptides and the capacity to hydrolyze dehydropeptides either occur together or (in other tissues) do not occur at all. This parallel feature lends support to the concept that the cystine peptides are degraded by the consecutive action of exocystine desulfurase and dehydropeptidase.

GASTRIC FUNCTION OF DOGS AFTER STIMULATION WITH ACETYL-β-METHYLCHOLINE CHLORIDE AND HISTAMINE DIPHOSPHATE

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During the course of an investigation which required data concerning the gastric function of dogs with stomach fistulas,[1] histamine diphosphate and acetyl-β-methylcholine chloride (mecholyl) were used as gastric stimulants. Since the literature contains few comparisons in the same animal of the effects of single injections of these gastric stimulants upon the secretion of acid and of pepsinogen, we felt that our results might be of some interest.

Babkin (*1*) concluded from his studies on dogs that histamine had no "trophic" effect on peptic and mucoid cells, or only a very insignificant one. Histamine was found to activate chiefly the parietal cells which are supposed to produce, in the juice, the hydrochloric acid or its precursors. The appearance of pepsin in the gastric juice secreted in response to injections of histamine was attributed by Babkin to a "washing out" effect of the juice. The conclusions of Gilman and Cowgill (*2*) were in accord with those of Babkin with regard to the effect of histamine upon gastric secretion. Using dogs with pouches of the Pavlov and Heidenhain type, they observed an increased volume of secretion and a relative decrease in pepsin concentration in response to histamine stimulation. Toby (*3, 4*), also investigating the effect of histamine stimulation upon gastric peptic activity in man and in dogs, reported that histamine did not augment the output of pepsin. She concluded that "histamine gastric secretion" reflects the state of activity of the peptic cells before the injection of the stimulant. In opposition to these views are those of Polland and co-workers (*5, 6*) and of Rivers et al. (*7, 8*), who observed that administration of histamine in man resulted in an increased output in pepsin. Gray and Ivy (*9*) reviewed the literature relating to the effect of acetylcholine upon gastric secretion and reported their own observations on the effects of mecholyl and of histamine phosphate in dogs that had stomach pouches. In their experiments, hydrochloric acid output was determined, either after repeated injections of mecholyl followed in some instances by single injections of histamine, or after repeated injections of histamine followed by single injections of mecholyl. Peptic activity was not determined. They demonstrated that mecholyl was potent in stimulating a flow of acid gastric secretion in dogs, and that with their procedure of repeated injections, the results indicated that molecule for molecule, histamine and mecholyl were of approximately equal potency. Gray and Ivy observed, furthermore, that supraoptimal doses of mecholyl produced salivation in the animals whereas optimal and suboptimal doses were without such action. They discussed the possible mechanism of the action of mecholyl. Necheles, Motel, Kosse, and Neuwelt (*10*) found mecholyl and histamine to be synergistic relative to gastric secretion of man and of dogs.

[1] The fistulas were made by Maj. Morris K. Barrett (M. C. A. U. S.,) now on leave from the National Cancer Institute.

EXPERIMENTAL PROCEDURE

A comparison was made of the gastric function of eight dogs with stomach fistulas in response to single injections of molecular equivalent doses of mecholyl and of histamine. The fistulas were prepared by the introduction, into the stomach, of a small segment of the dog's own small intestine and the attachment of the other end of the piece of intestine to the muscle surrounding a small opening in the abdominal wall. These stomach fistulas, thus made of intestine, were placed in such a manner that the stomach contents did not ooze. The animals were maintained under identical housing and feeding conditions. They are mon-

grels, weighing from 11.5 to 19.0 kg. and with one exception, Brownie, have appeared well-nourished throughout the period of observation. Before preparation of the fistulas, and during an experimental period of 18 months following the introduction of the fistulas, gastric function was determined at frequent intervals after histamine stimulation, and the response remained remarkably constant in the individual dogs.

Between the fifteenth and twenty-third months following the introduction of the fistulas, 72 gastric function tests were made, 5 after mecholyl chloride stimulation, and 4 following histamine diphosphate with each dog. The animals were fasted for 18 to 20 hours before each test. Determination was made of the least amount of mecholyl chloride, which, in a single subcutaneous injection, would elicit an optimum gastric response. Doses of 0.000125, 0.00025, and 0.0005 milliequivalent of mecholyl chloride per kilogram of body weight were tried with each dog. A comparison was made then of gastric function responses of all the dogs to the optimum dose and to half this dose of mecholyl and to equivalent amounts of histamine. Samples of juice were collected at 15-minute intervals by aspiration from the stomach through the fistula, by using a catheter to which was attached a 50-cc. syringe. Volume of juice, free hydrochloric acid, total acid, and peptic activity were measured in each sample after passing the juice through a fine-mesh gauze filter.

ANALYSIS OF GASTRIC JUICE

Töpfer's indicator (dimethylaminoazobenzene) and phenolphthalein were used for the titration of free hydrochloric acid and total acid, respectively. Peptic activity was measured by a modification of the Anson method (*11*). One-cubic-centimeter samples of suitable dilutions of the gastric juice were used for the digestion of the acidified hemoglobin substrate during a 10-minute digestion period, at a temperature of 37° C. The 2.5-percent aqueous-hemoglobin solution was prepared fresh from lyophylized horse hemoglobin. The hemoglobin after extraction from the centrifuged clot, was washed, dialyzed, frozen rapidly in a dry ice-cellosolve mixture, and dehydrated in a Cryochem apparatus. Color comparisons were made in a Universal Spectrophotometer with the use of a standard tyrosine curve. Suitable dilutions of the gastric juice, made with 0.2 N hydrochloric acid, varied from 1:50 to 1:200 (more often 1:200). The same dilution was made of all the fractions in a single test since it was found that an increase in dilution resulted in a slight increase in the relative amount of color produced. Peptic activity is expressed as milligrams of tyrosine liberated per minute from the hemoglobin substrate by 100 cc. of undiluted gastric juice.

RESULTS

In figure 1, graphs 1 and 2 present composite curves of total output, and graphs 3 and 4 present the composite curves for concentrations of the various gastric constituents of the 15-minute samples for all the dogs after histamine and mecholyl stimulation. Fasting juice was present in the individual dogs so infrequently and irregularly that the analyses were not included in the composite curves. In all fasting samples, there was little or no free acid, but often a higher concentration of peptic activity than in the juice secreted in response to a stimulant. Although there was variation in the response of the individual dogs to each dose of each stimulant, the similarity was great enough, especially with the 0.000125 and 0.00025 milliequivalent doses, to permit composite curves to represent accurately the responses of the individuals. The constant response of each dog to a given dose of a stimulant at different times was striking.

As indicated in graph 2, the middle dose of mecholyl (0.00025 milliequivalent per kilogram of body weight) ef-

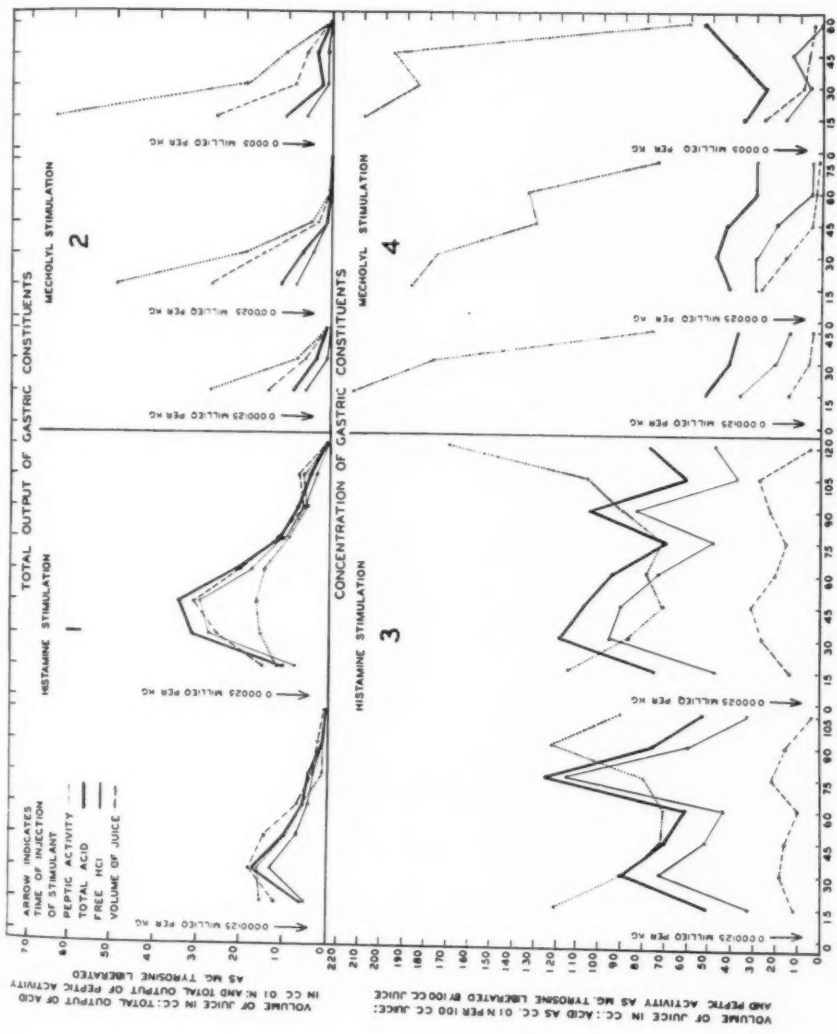
TOTAL OUTPUT OF GASTRIC CONSTITUENTS
CONCENTRATION OF GASTRIC CONSTITUENTS
HISTAMINE STIMULATION
MECHOLYL STIMULATION
1
2
3
4
ARROW INDICATES TIME OF INJECTION OF STIMULANT
PEPTIC ACTIVITY
TOTAL ACID
FREE HCl
VOLUME OF JUICE
0.00125 MILLIG PER KG
0.0025 MILLIG PER KG
0.005 MILLIG PER KG
MINUTES
VOLUME OF JUICE IN CC.: TOTAL OUTPUT OF ACID IN CC .01 N. AND TOTAL OUTPUT OF PEPTIC ACTIVITY AS MG. TYROSINE LIBERATED
VOLUME OF JUICE IN CC.: ACID AS CC. .01 N PER 100 CC. JUICE: AND PEPTIC ACTIVITY AS MG. TYROSINE LIBERATED BY 100 CC. JUICE

FIGURE 1.—Composite curves showing the gastric responses of 8 dogs to histamine and to mecholyl stimulation.

fected a greater gastric response than did the lower dose of 0.000125 milliequivalent per kilogram. This was the case for each dog. Six of the dogs secreted more gastric juice after the middle dose of mecholyl than after the largest dose of 0.0005 milliequivalent per kilogram of body weight; one dog gave similar results with both the middle and the high doses; and one dog secreted more juice of exceedingly high acidity and peptic activity following the highest dosage. There was salivation following all the doses of mecholyl; even the lowest dose, which amounted to a single injection of 0.28 to 0.48 mg. per dog and which was suboptimal in its stimulating effect, produced salivation for the first 20 minutes following administration. This finding is different from that of Gray and Ivy (*9*), who observed that optimal and suboptimal doses of mecholyl did not produce salivation. After the 0.000125 and 0.00025 milliequivalent doses, the first two samples of juice (which represented most of the total secretion of juice after mecholyl) contained a thick layer of foamy saliva which would not pass through the gauze filter and which, therefore, separated easily from gastric juice that readily passed through the filter. The juice secreted in response to the 0.0005 milliequivalent dose, on the other hand, was viscous throughout and was sometimes difficult to pipette. Since the middle dose of 0.00025 milliequivalent of mecholyl seemed to be preferable in six of the dogs, a comparison was made of the gastric response of each animal to stimulation by 0.00025 milliequivalent of histamine. The responses of all the animals to half this dose of histamine were also determined. As the composite curves in graph 1 demonstrate, the dogs gave a greater response to the 0.00025 milliequivalent dose of histamine than to the lower dosage.

The graphs show clearly that single injections of histamine (graphs 1 and 3) effect a different stimulation of the gastric mucosa than do equivalent doses of mecholyl (graphs 2 and 4). After a single subcutaneous injection of 0.00025 milliequivalent of histamine per kilogram of body weight, juice was secreted for a longer period of time, a larger volume of juice resulted, and the free acid and total acidity were greater than after the equivalent dose of mecholyl chloride. The possibility cannot be overlooked, however, that contamination of the juice by saliva and/or an increased mucin content of the gastric juice after mecholyl stimulation may have been instrumental, in part at least, in neutralizing some of the acid and thus may have resulted in a lower acid content than after histamine stimulation.

After histamine, the volume of juice and the total outputs of acid and of pepsin increased in the successive 15-minute samples until they reached a maximum 30 to 45 minutes after the injection of the stimulant (graph 1). As illustrated in graph 3, the concentration of acid and the volume of juice showed two peaks, and there was no apparent correlation between pepsin concentration and the concentration of acid. The total output of peptic activity, however, showed a correspondence to acid and volume outputs. The fact that the rate of output of peptic activity after histamine showed a similarity to the rate of output of juice and of acid whereas the concentration of peptic activity was not correlated with the concentration of acid suggests that the correspondence in output was the result of a "washing out" effect, as described by Babkin (*1*).

After mecholyl stimulation, there appeared to be a definite correspondence between both the total outputs (graph 2) and the concentrations (graph 4) of free hydrochloric acid, total acid and peptic activity, the peptic activity showing an exaggerated response at the peak of secretion. The maximum volumes of juice, the highest concentrations of acid, and the greatest outputs of juice, of acid and of pepsin were found usually in the sample that was

collected 15 minutes after the injection of mecholyl. The average concentration of peptic activity per cubic centimeter of juice secreted per minute by the eight dogs was 5 times as high after the 0.000125 dose and 1.5 times as high after the 0.00025 dose of mecholyl than after equivalent doses of histamine. The total output of the enzyme activity, however, was 25 percent less after the lowest dose of mecholyl than after the equivalent dose of histamine, and was practically the same after both stimulants in response to the 0.00025 milliequivalent dose. The peptic activity after mecholyl stimulation appeared largely during the first 15 minutes; following histamine, on the other hand, juice was secreted for 60 to 120 minutes, and peptic activity was present in all the samples collected. A comparison of the combined total outputs of the gastric constituents of the eight dogs in response to the 0.00025 milliequivalent dose of each stimulant is given in table 1.

TABLE 1.—*The combined outputs of gastric constituents of 8 dogs secreted in response to single doses of 0.00025 milliequivalents of histamine and of mecholyl*

Gastric constituent	After histamine stimulation	After mecholyl stimulation	Ratio of total amounts secreted	Ratio of total amounts secreted per minute
			Histamine: Mecholyl	Histamine: Mecholyl
Juice.....cc..	954	382	2.5:1	1.3:1
Free hydrochloric acid.....cc. 0.1N..	798	110	7.2:1	3.9:1
Total acid.....cc. 0.1N..	957	167	5.7:1	3.1:1
Peptic activity.....mg. tyrosine..	637	622	1.0:1	.55:1
Duration of secretion.....minutes..	630 [1] (60–120)	345 [1] (30–75)	1.8:1	----------

[1] Limits of duration of secretion in minutes for individual dogs.

SUMMARY

In eight dogs having stomach fistulas, single subcutaneous doses of 0.00025 milliequivalent per kilogram of body weight of either mecholyl chloride or of histamine diphosphate were found to be effective as stimulants of gastric secretion. After mecholyl, this dosage appeared to be the optimum of the three doses studied (0.000125, 0.00025, and 0.0005 milliequivalent per kilogram of body weight).

Juice was secreted for periods of 30 to 75 minutes in response to the optimum dose of mecholyl, the maximum responses occurring usually during the first 15 minutes, and from 60 to 120 minutes after an equivalent dose of histamine, the maximum responses occurring between 30 and 45 minutes.

The gastric juice secreted following mecholyl stimulation had a higher pepsin concentration and a lower acid content than did the juice secreted in response to equivalent doses of histamine.

The total outputs of acid and of juice were less after mecholyl than after equivalent doses of histamine. Although the rate of secretion of pepsin was greater after mecholyl, the total outputs of peptic activity were the same after doses of 0.00025 milliequivalent per kilogram body weight of mecholyl and of histamine.

Mecholyl chloride caused salivation after the suboptimal, the optimal, and the supraoptimal doses, but the highest dosage used, 0.0005 milliequivalent per kilogram of body weight, produced the most profuse salivation.

The experimental results support the theory that mecholyl stimulates the secretion of both pepsin and acid by the gastric mucosa, and that the major effect of histamine upon the gastric mucosa is the stimulation of the secretion of juice and of acid.

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